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(54) Title: MONO- AND BIFUNCTIONAL MOLECULES WITH ABILITY TO BIND TO G PROTEIN-COUPLED RECEPTORS

(57) Abstract: The invention discloses chemokine fusion proteins comprising at least one chemokine or chemokine variant and a polypeptide comprising one optionally modified portion of the constant region of an immunoglobulin molecule having the ability to bind to the corresponding chemokine receptor and to inhibit cell migration induced by the presence of native or wild type chemokines. These fusion proteins may be used to diagnose or treat diseases or conditions characterized by the presence or migration of immune or inflammatory cells. Pharmaceutical compositions comprising these fusion proteins are also disclosed.

MONO- AND BIFUNCTIONAL MOLECULES WITH ABILITY TO BIND TO G PROTEIN-COUPLED RECEPTORS

DESCRIPTION

Field of the Invention

[0001] The invention relates to fusion proteins comprising a chemokine or a variant thereof and a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin with the ability to bind to the corresponding chemokine receptor and inhibit the cell migration induced in the presence of the specific native chemokine. Said fusion proteins can be used with therapeutic, diagnosis or research purposes, among others.

Background of the Invention

[0002] Leukocyte traffic is a critical process for immune response. Controlled cell movements allow the precise interactions necessary for putting different types of leukocytes into physical contact. The possibility that T and B lymphocytes find their specific antigen is maximized when these lymphocytes are recirculated through secondary lymphatic organs, in which antigen-presenting cells are shown. In the last few years, the data from different scientific publications indicate that chemokines or factors with chemoattractant activity are important factors in controlling leukocyte traffic.

[0003] Chemokines are a family of chemoattractant proteins with a low molecular weight (8-15 kDa) involved in leukocyte activation and migration. These proteins are responsible for coordinating leukocyte traffic in inflammatory processes through the activation of specific heterotrimeric G protein-coupled receptors, inducing the cells to migrate towards the inflammation site in favor of a concentration gradient. In addition to its role in recruiting leukocytes, it is currently known that chemokines carry out important functions in cell proliferation and apoptosis, tissue morphogenesis, hematopoiesis, as well as in angiogenesis and in

the development of specific immune responses. Likewise, the involvement of chemokines and their receptors in autoimmune diseases and in the infection due to the human immunodeficiency virus (HIV) has also been described.

[0004] All the molecules belonging to the chemokine family share structural characteristics. Their three dimensional structure has three β-pleated sheets (denominated β1, β2 and β3) connected by loops, a loop in the N-terminal region and an α helix in the C-terminal domain. Its carboxyl end has a basic nature and has an affinity for binding to compounds such as heparin, glycosaminoglycans, carbohydrate molecules and negatively charged sulphated proteins which are found in the cell surface and in the extracellular matrix.

[0005] Most chemokines have four cysteine residues in highly conserved positions and joined to each other by disulphide bridges. The relative position of the first two cysteines in the N-terminus allows their classification in four subfamilies: CXC, CC, XC and CX3C. Within these subfamilies, the main ones are denominated CXC and CC chemokines; the first ones are characterized by having two cysteine residues separated by any amino acid whereas in CC chemokines, both cysteine residues are adjacent. The other two subfamilies described are CX3C, characterized by having three amino acids between the first two cysteines, and XC lacking cysteine in the most N-terminal position.

[0006] Chemokines carry out their action by means of the activation of specific receptors, "chemokine receptors", belonging to the superfamily of heterotrimeric G protein-coupled receptors (GPCRs). The receptors of this family have seven transmembrane domains and their structure has three intracellular loops, three extracellular loops, an extracellular N-terminus end and an intracellular C-terminus end. The extracellular region of the receptor participates in the binding to the ligand, whereas the

intracellular region is involved in signal transduction (Horuk R. 1994). There are at least sixteen human chemokine receptors that bind or respond to chemokines with the following characteristic model: CCR-1 [MIP-1α, MIP-1β, MCP-3, RANTES, MCP-1, HCC-1, HCC-4] (Ben-Barruch. et al., 1995); (Neote, et al., 1993); CCR-2A and CCR-2B (or "CKR-2A"/"CKR-2A" or "CC-CKR-2A"/"CC-CKR-2A") [MCP-1, MCP-2, MCP-3, MCP-4]; CCR-3 (or "CKR-3" or "CC-CKR-3") [eotaxin, eotaxin 2, RANTES, MCP-3, MCP-4, MCP-2, MTP-18] (Combadiere. et al., 1995); CCR-4 (or "CKR-4" or "CC-CKR-4") [(MIP-1α, RANTES, MCP-1] (Power, et al., 1995); CCR-5 (or "CKR-5" OR "CC-CKR-5") [MDC, TARC] (Samson et al., 1996); CCR-6 (LARC), MIP-3α; CCR-7 (6C-kine, MIP-3β); CCR-8 (I-309, LEC); CCR-9 (TECK); CCR-10 (CTAK); CCR-11 (ELC, SLC, TECK); CXCR-1 (NAP-2, GCP-2, IL-8; CXCR-2 (GROα; GROβ, GROγ;, ENA-78, NAP-2, IL-8, GCP-2); CXCR-3 (MIG, IP-10, I-TAC); CXCR-4 (SDF-1α, SDF-1β); CXCR-5 (BLC), CXCR-6 (CXCL-16); XCR1 (SCM-1β, SCM-1α); CX3CR1 (fractalkine) and the Duffy blood group antigen [RANTES, MCP-1] (Chaudhuri, 1994). In the last few years, a standard nomenclature has been established for chemokines, as explained in Murphy PM, 2002.

[0007] The chemokine system has a certain promiscuity in the sense that there are chemokines capable of binding to more than one receptor and receptors capable of interacting with more than one ligand or chemokine. An interpretation of this fact is that the redundancy of contacts between different chemokines and a receptor or vice versa, provides robustness to the system which in this way can work in the absence of some of its constituent elements. Alternatively, the redundancy in the binding to the receptor can represent a hierarchy in the chemokines with binding affinities for the receptor from strongest to weakest, the different chemokines being capable of working in an orderly manner in order to better direct the leukocyte traffic. The fact that chemokines have a high degree of redundancy makes it potentially difficult to

produce a specific anti-inflammatory effect by blocking a single chemokine receptor, because the inhibition of a single element of the system may be compensated by another element, decreasing the effectiveness of the therapeutic intervention. Nevertheless, it has been observed that there are different levels of control in the chemokine system allowing the existence *in vivo* of an assignment of specific and non-redundant functions.

[0008] On the other hand, there are data indicating that the signaling by chemokines can integrate cytokine signaling molecules but the specific characteristics of GPCR signaling are maintained. The activation of this signaling pathway through GPCRs sets forth some questions. The classic opinion of JAK/STAT activation by cytokine receptors requires the presence of two JAK that are close, such that they can be activated by trans-phosphorylation. In the case of cytokine receptors, this is achieved by means of the dimerization of the receptors which have been associated in a constitutive manner to JAK. Dimerization also occurs in the case of chemokines. In this aspect, the dimerization of chemokine receptors was first shown for CCR2 using different experimental approaches which included the use of agonist monoclonal antibodies, labeled receptors, mutant receptors and energy transfer tests. This was later generalized to other chemokine receptors (such the CCR5 and CXCR4).

[0009] Physiologically, chemokines do not act in an isolated manner but several chemokines act in a coordinated manner during homeostasis and also in inflammatory processes. It has recently been observed that the simultaneous stimulation with CCL2 and CCL5 induces the formation of the heterodimer CCR2-CCR5 (Mellado et al., 2001). The heterodimeric chemokine receptor has exceptional characteristics such the reduction of the threshold of response to chemokines, and the flow of Ca^{2+} independent of the pertussis toxin (PTx), which indicates properties different from those of the homodimer of said receptors. The heterodimer promotes the specific

recruitment of GqIII, explaining the flow of Ca^{2+} resistant to PTx, affecting the subunits GαI and it also shows differences in PI3K activation and increase of cell adhesion. Furthermore, the heterodimer annuls receptor internalization, indicating that there is no decrease of receptor levels in the surface. An implication of receptor clustering as a consequence of chemotactic responses is that the activity of a receptor affects that of its neighbors, such that a ligand can act in *trans* in a receptor for which it is not specific. In this way, the bound ligand can induce changes in the receptor signaling activity and these changes are propagated to a large number of neighboring receptors, amplifying the effect of a single binding event. The sensitivity of a receptor can be regulated by the formation of signaling domain complexes qualitatively dictated by the availability of chemokines. Therefore, dimerization can be a critical step in physiological situations in which chemokines are involved, including collaborating T cell responses, hematopoiesis, angiogenesis and homeostasis, as well as in pathological conditions in which inflammatory processes such asthma, tumor rejection, and arteriosclerosis intervene or in pathologies caused by HIV-1 infection, in which it has been observed that the dimerization of chemokine receptors directly prevent infection due to HIV-1.

[0010] Compounds and/or molecules capable of inhibiting the chemotactic signal or the cell blocking of specific chemokine receptors allows dealing therapeutically and specifically with processes and pathologies in which cell migration is mediated by said receptors. An advantage of acting by inhibiting the chemoattractant activity of chemokines arises from the fact that said inhibition allows preventing the formation of leukocyte infiltrates in inflammatory processes, instead of acting on these infiltrates like the anti-inflammatory drugs currently used in clinical medicine. In this sense, numerous molecules have been developed including specific antagonist antibodies of chemokine

receptors, small chemical molecules with antagonist activity, RNA aptamers, peptides, inhibitory peptide mimics, antisense oligonucleotides, polymers and variant antagonists derived from native chemokines. Several studies have been described relating the structure-activity of chemokine variants obtained by means of introducing modifications in the N-terminus of the protein either by deletion or extension of amino acids or by chemical modification on the N-terminal residue of the chemokine and which reduce the activity and/or affinity of the native chemokine for the receptor and/or act as antagonist molecules, for example for: CCL2 (Gong JH et al., 1995; Zhang et al., 1995; Beall CJ et al., 1996; Steitz SA et al., 1998; Gu L et al., 1999; Hemmerich S et al., 1999; Seet BT et al., 2001) and eotaxin (Mayer MR and Stone MJ, 2001).

[0011] The half-life of the chemokine variants is short in comparison to the one generally required for the use of an active ingredient in therapeutics. The fusion of an immunoglobulin Fc fragment to a protein increases the production and secretion thereof and facilitates its purification. Said Fc fragment is very soluble and relatively large, such that it allows potentially extending the circulating half life of pharmacological proteins *in vivo*, protecting them from degradation and allowing a slower biological clearance. An increase in the half-life time of a therapeutically effective molecule allows reducing the number of administrations thereof.

[0012] Gutiérrez J. et al., 2004, describes fusion proteins of the Chemokine-Fc type which were exclusively used as a tool for monitoring the binding of the mouse CCL1 ligand to a collection of murine CCR8 receptor mutants in order to carry out structure-function studies. Krautwald S. et al., 2004, describes a fusion protein formed by a mouse CCL19 chemokine fused to an Fc fragment of the mouse IgG_{2b} immunoglobulin, with the general formula $(CCL19\text{-}Fc)_2$ having an ability to bind to the murine CCR7 receptor similar

to that of the native ligand (CCL19).

[0013] Nevertheless, there is still a need to develop compounds capable of inhibiting the binding of a chemokine to its receptor thus causing an inhibition of the chemotactic signal or the cell blocking of specific chemokine receptors. Said compounds would allow to therapeutically and specifically deal with processes and pathologies in which cell migration is mediated by said receptors.

Summary of the Invention

[0014] The present invention describes the development of fusion proteins comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin having the ability to bind to the corresponding chemokine receptor and surprisingly, having the ability to inhibit the cell migration *in vitro* induced in the presence of the specific native (wild type) chemokine. The fusion proteins of the present invention can be used in the therapeutic treatment of processes and pathologies in which cell migration is mediated by chemokine receptors, for example, in the treatment of inflammatory, autoimmune diseases, cancer and infections of a bacterial, protozoal, or viral origin.

[0015] Accordingly, in a primary aspect of the invention, a fusion protein is provided that comprises (i) a chemokine and (ii) a polypeptide Y comprising a portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y) - (Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein at least one of (i) or (ii) is a non-wild type, or a variant as defined herein.

[0016] In a further aspect of the invention, a fusion protein is provided comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein Q_A and Q_B are particular chemokines, or chemokine variants, defined in the particular embodiments described below, and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin, also described in the particular embodiments described below.

[0017] In one particular embodiment, Q_A is CCL2 or a CCL2 variant, preferably a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region;
Q_B is a chemokine or a chemokine variant different from Q_A; and
Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0018] In another particular embodiment, Q_A is CCL5 or a CCL5 variant, preferably a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region;
Q_B is a chemokine or a chemokine variant different from Q_A; and
Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0019] In yet another particular embodiment, Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A);
Q_B is a chemokine or a chemokine variant different from Q_A; and
Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0020] In yet another particular embodiment, Q_A is a variant of the CCL5 chemokine, designated CCL5(S24MP25A);
Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0021] In yet another particular embodiment, Q_A is a CC chemokine selected from the group consisting of: CCL2, CCL5, CCL3 and CCL4 or variants thereof;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0022] In yet another particular embodiment, Q_A is a variant of a CC chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A), CCL2(Y13A) and CCL5(S24MP25A);

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0023] In yet another particular embodiment, Q_A is a CXC chemokine selected from the group consisting of: CXCL12 and CXCL8 or variants thereof;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0024] In yet another particular embodiment, the fusion protein comprises (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

wherein

Q_A is CCL2 or a CCL2 variant, preferably a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is CCL5 or a CCL5 variant, preferably a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0025] In yet another particular embodiment, the fusion protein comprises (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

wherein

Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A);

Q_B is a variant of the CCL5 chemokine, designated CCL5(S24MP25A); and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0026] A second aspect of the invention provides a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula $(Q_A\text{-}Y)_2$, with the condition that:

- when Q_A is CCL1 from mouse, then Y is not the fragment Fc of the human IgG_1 immunoglobulin; and
- when Q_A is CCL19 from mouse, then Y is not the fragment Fc of the mouse IgG_{2b} immunoglobulin;

wherein

Q_A and Y have the meanings indicated previously.

[0027] In one particular embodiment, the fusion protein has the

general formula $(Q_A-Y)_2$, in which Q_A is a CC chemokine or a variant thereof or a CXC chemokine or a variant thereof; and the polypeptide Y comprises a native Fc fragment.

[0028] In another particular embodiment, Q_A is a CC chemokine selected from the group consisting of CCL2, CCL5, CCL3 and CCL4, or a variant thereof.

[0029] In yet another particular embodiment, Q_A is a variant of CCL2 selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or a variant of CCL5, designated CCL5(S24MP25A).

[0030] In yet another particular embodiment, Q_A is a CXC chemokine selected from the group consisting of CXCL12 and CXCL8, or a variant thereof.

[0031] In yet another particular embodiment, the chemokine or chemokine variant has the ability to interact with at least one of the cell surface receptors CCR2 and/or CCR5.

[0032] In yet another particular embodiment, the polypeptide Y comprises a portion of the constant region of an immunoglobulin molecule

[0033] In yet another particular embodiment, the immunoglobulin molecule is of human origin.

[0034] In yet another particular embodiment, the polypeptide Y comprises at least one domain of the constant region of the heavy chain of an immunoglobulin molecule.

[0035] In yet another particular embodiment, the polypeptide Y

comprises at least one domain of the heavy chain of an immunoglobulin selected from the CH2 domain and the CH3 domain of the heavy chain of an immunoglobulin.

[0036] In yet another particular embodiment, the polypeptide Y comprises the CH2 and CH3 domains of the heavy chain of an immunoglobulin.

[0037] In yet another particular embodiment, the polypeptide Y further comprises the hinge region of an immunoglobulin or a fragment thereof.

[0038] In yet another particular embodiment, the polypeptide Y comprises a portion of the constant region of an IgG isotype immunoglobulin selected from IgG1, IgG2, IgG3 and IgG4.

[0039] In yet another particular embodiment, the portion of the constant region of an immunoglobulin is an IgG1 isotype immunoglobulin.

[0040] In yet another particular embodiment, the fusion protein comprises the general formula $(Q_A\text{-}Y)_2$, in which Q_A is a variant of CCL2 selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or a variant of CCL5, designated CCL5(S24MP25A); and the polypeptide Y comprises a native Fc fragment.

[0041] In yet another particular embodiment, the constant region of the immunoglobulin consists of an Fc variant which has been modified such that the binding to a specific Fc receptor is prevented.

[0042] In yet another particular embodiment, the Fc variant is an Fc variant of a human IgG1 immunoglobulin.

[0043] In yet another particular embodiment, the Fc variant has a substitution mutation of one or more amino acids, selected from the group consisting of E233P, L234V and L235A.

[0044] In yet another particular embodiment, the fusion protein is a monomer of general formula Q_A-Y in which Q_A is a CCL2 variant selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or is a variant of CCL5, designated CCL5(S24MP25A).

[0045] In yet another particular embodiment, the fusion protein comprises the general formula Q_A-Y, in which Q_A is a chemokine or a variant thereof, and wherein the variant is either a CCL2 variant selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or is a variant of CCL5, designated CCL5(S24MP25A); and wherein the polypeptide Y comprises an Fc variant either lacking or having modifications in the residues involved in the formation of disulphide bridges.

[0046] In yet another particular embodiment, the Fc variant is an Fc variant of a human IgG1 immunoglobulin.

[0047] In yet another particular embodiment, the Fc variant has a mutation in which the cysteine at positions 226 and/or 229 has been replaced with a serine and/or an alanine.

[0048] In yet another particular embodiment, the polypeptide Y comprises an Fc variant which has been modified such that it favors the formation of heterodimers.

[0049] In yet another particular embodiment, the Fc variant is an Fc variant of a human IgG1 immunoglobulin.

[0050] In yet another particular embodiment, the modification consists of a "knob into hole" mutation comprising mutations in the knob chain selected from the group consisting of Y349C and T366W, and mutations in the hole chain selected from the group consisting of D356C, T366S, L368A and Y407V.

[0051] In yet another particular embodiment, the fusion protein is a heterodimer of general formula (Q_A-Y)(Q_B-Y), in which Q_A is CCL2 or a variant thereof selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), and Q_B is CCL5 or a variant thereof, designated CCL5(S24MP25A).

[0052] In yet another particular embodiment, the fusion protein is a monofunctional dimer of general formula Q_A-Y_2, in which Q_A is CCL2 or a variant thereof selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or CCL5 or a variant thereof designated CCL5(S24MP25A).

[0053] In yet another particular embodiment, the fusion protein further comprises a spacer peptide between the chemokine or a variant thereof and the polypeptide Y.

[0054] In yet another particular embodiment, the spacer peptide comprises the entire or a portion of the hinge region of an immunoglobulin.

[0055] In yet another particular embodiment, the fusion protein further comprises a tag peptide.

[0056] In yet another particular embodiment, the fusion protein further comprises a signal peptide.

[0057] In yet another particular embodiment, the fusion proteins of the invention provide for an increased inhibitory, antagonistic, or blocking effect on the normal activity or function of a native or wild type chemokine. Accordingly, the fusion proteins of the invention, particularly the fusion proteins comprising a chemokine or a chemokine variant and an optionally modified Fc region of an immunoglobulin molecule, bind to the native chemokine receptors, and lack signaling capability. Thus, these fusion proteins provide for an effective means of treating diseases or conditions caused in part, by leukocyte migration and the ensuing inflammation associated with the condition and also treating and/or preventing protozoal or viral infections, for example human inmunodeficiency virus infection The presence of the optionally modified portion of the immunoglobulin molecule allows for a longer half-life of the molecule. Thus, the fusion proteins of the invention have several advantages over the variants known to those skilled in the art.

[0058] A third aspect of the invention provides a nucleic acid molecule encoding a fusion protein as described in any of the embodiments noted herein.

[0059] A fourth aspect of the invention provides a vector comprising a nucleic acid molecule encoding a fusion protein as described in any of the embodiments herein.

[0060] A fifth aspect of the invention provides a host cell containing a vector comprising a nucleic acid molecule encoding a fusion protein as described in any of the embodiments noted herein.

[0061] A sixth aspect of the invention provides a process for obtaining a fusion protein with activity inhibiting the binding of

a chemokine to a specific receptor thereof *in vitro*, comprising the following steps:

a) generating a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin;

b) testing the ability of said fusion protein to inhibit the binding of said chemokine to a specific receptor thereof; and

c) selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro*.

[0062] A seventh aspect of the invention provides a process for selecting a fusion protein having activity inhibiting the binding of a native chemokine to a specific receptor thereof *in vitro*, improved with respect to a variant of said chemokine, comprising the following steps:

a) generating a fusion protein comprising (i) a variant of a native chemokine and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin;

b) testing the ability of said fusion protein to inhibit the binding of said native chemokine to a specific receptor thereof; and

c) selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro* greater than said chemokine variant.

[0063] An eighth aspect of the invention provides a pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an

immunoglobulin, of general formula:

(a) $(Q_A-Y)-(Q_B-Y)$;

(b) Q_A-Y;

(c) $Q_A-(Y)_2$ and

(d) $(Q_A-Y)_2$

wherein Q_A and Q_B are particular chemokines, chemokine variants or fragments thereof, defined in the particular embodiments described herein, and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin, also described in the particular embodiments described herein, and a pharmaceutically acceptable carrier.

[0064] In one particular embodiment of the composition, Q_A is CCL2 or a CCL2 variant, preferably a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region; Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0065] In another particular embodiment of the composition, QA is CCL5 or a CCL5 variant, preferably a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region; Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0066] In yet another particular embodiment of the composition, Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A); Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0067] In yet another particular embodiment of the composition, Q_A is a variant of the CCL5 chemokine designated CCL5(S24MP25A); Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0068] In yet another particular embodiment of the composition, Q_A is a CC chemokine selected from the group consisting of: CCL2, CCL5, CCL3 and CCL4 and variants thereof; Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0069] In yet another particular embodiment of the composition, Q_A is a CXC chemokine selected from the group consisting of CXCL12, CXCL8, and variants thereof; Q_B is a chemokine or a variant thereof, different from Q_A; and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0070] In yet another particular embodiment of the composition, Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A); Q_B is a variant of the CCL5 chemokine, designated CCL5(S24MP25A); and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin molecule.

[0071] A ninth aspect of the invention provides a pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula $(Q_A\text{-}Y)_2$ and a pharmaceutically

acceptable carrier, with the condition that:

a) when Q_A is CCL1 from mouse, then Y is not the fragment Fc of the human IgG_1 immunoglobulin; and

b) when Q_A is CCL19 from mouse, then Y is not the fragment Fc of the mouse IgG_{2b} immunoglobulin;

wherein Q_A and Y have the meanings indicated previously.

[0072] In one particular embodiment, the pharmaceutical composition further comprises other additional drugs having anti-inflammatory, antitumor, antiprotozoal or antiviral activity. These other drugs are administered simultaneously or sequentially.

[0073] A tenth aspect of the invention provides for the use of a fusion protein in the manufacture of an anti-inflammatory, antitumor, antiprotozoal or antiviral pharmaceutical composition.

[0074] In one particular embodiment, the pharmaceutical composition is an antiviral pharmaceutical composition.

[0075] In another particular embodiment, the pharmaceutical composition is a pharmaceutical composition for the treatment of the infection caused by the human immunodeficiency virus.

[0076] In yet another particular embodiment, the pharmaceutical composition is a pharmaceutical composition for the treatment of inflammatory or autoimmune diseases in which chemokine receptors are involved.

[0077] An eleventh aspect of the invention provides for the use of a fusion protein in the manufacture of a composition for the diagnosis *in vitro or in vivo* of diseases in which the levels of a certain chemokine are altered.

[0078] In one particular embodiment, the fusion protein is used in the qualitative or quantitative detection of the presence of chemokine receptors on the surface of a cell *in vitro*.

[0079] A twelfth aspect of the invention provides for the use of a fusion protein in the identification of a compound with the ability to compete with said fusion protein for binding to a chemokine receptor *in vitro*.

[0080] A thirteenth aspect of the invention provides a method of treating a disease or condition characterized, in part, by the presence of cells bearing a chemokine receptor, comprising administering an effective amount of a fusion protein as described above to a mammal in need of such therapy.

[0081] In one particular embodiment, the disease or condition is selected from the group consisting of an inflammatory disease, a cancerous condition, a protozoal infection or a viral condition.

[0082] In another particular embodiment, the viral infection is caused by a lentivirus. In one particular embodiment, the lentivirus is human immunodeficiency virus.

[0083] The uses and aspects of the fusion proteins will be better understood in the description of the invention.

Brief Description of the Figures

[0084] Figure 1 shows the basic structure of the human IgG1 immunoglobulin consisting of 4 polypeptide chains joined to each other by disulphide bridges. They are identical two-by-two chains, the so-called heavy chains (H) and light chains (L). Likewise, an immunoglobulin molecule consists of a variable region (V) and a constant region (C). The three-dimensional structure of said

polypeptide chains is formed by several structural domains. The domains forming part of the heavy chain are denominated V_H, C_H1, C_H2 and C_H3, whereas the light chain is formed by the V_L and V_H domains.

[0085] Figure 2 shows schematically the structure of the fusion proteins of the invention. Figure 2A shows a diagram of the fusion protein of the invention of general formula $(Q_A\text{-}Y)_2$. Figure 2B shows a diagram of the fusion protein of the invention of general formula $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$. Figure 2C shows a diagram of the fusion protein of the invention of general formula $Q_A\text{-}(Y)$. Figure 2D shows a diagram of the fusion protein of the invention of general formula $Q_A\text{-}(Y)_2$.

[0086] Figure 3 shows photographs of a Western Blot carried out on the transfection supernatants collected 24, 48 and 72 hours after the transfection of HEK-293 cells. Figure 3A shows the expression of the fusion protein MCP1Fc in the supernatants collected from cells transfected with the pCCL2-Fc plasmid. Figure 3B shows the expression of fusion protein CCL5-Fc. In both figures, it can be observed that the apparent molecular weight of the expressed proteins corresponds to the theoretical weight of the dimer which is to be formed.

[0087] Figure 4 shows the photographs of a Western Blot carried out in samples collected in the different purification steps of the fusion proteins. In said assay, it is verified that the apparent molecular weight of the fusion proteins corresponds to the formation of the dimer. Figure 4A shows the purification steps of the fusion protein CCL2-Fc; in it, the protein expressed in the supernatant before being loaded in the purification column (pre); a sample of the supernatant after being loaded in the column (post) (in which it can be observed that the fusion protein is adhered to the G protein column); two samples of the purified fusion protein once it has been eluted and concentrated (pur), and a sample collected from the washings carried out in the column after it has been loaded (was), in which it is observed that it

does not carry the protein CCL2-FC along when the washing is carried out. Figure 4B shows the purification steps of the fusion protein CCL5-Fc, as explained in the case of CCL2-Fc. Figure 4C shows the purification steps of the fusion protein CCL3-FcR(-); Figure 4D shows the purification steps of the fusion protein CCL4-FcR(-), Figure 4E shows the purification steps of the fusion protein CXCL8-FcR(-) and Figure 4F shows the purification steps of the fusion protein CXCL12-FcR(-).

[0088] Figure 5 shows the results of a migration experiment carried out with THP1 cells to prove the specificity of the fusion protein CCL2-Fc. It is observed that the THP1 cells migrate against different concentrations of CCL2-Fc (5-20 nM) and this migration decreases if the THP1 cells that will migrate are incubated with 0.5 μg of anti-CCR2 antibody (1D9). The same graph shows that the incubation of the protein CCL2-Fc with an anti-MCP1 antibody decreases the migration, indicating a specific blocking of the chemokine part of the fusion protein CCL2-Fc.

[0089] Figure 6 shows the results of a calcium mobilization assay which shows the specificity of the fusion protein CCL2-Fc to the receptor CCR2. Figure 6A shows how the THP1 cells release calcium in response to the fusion protein CCL2-Fc (5 nM). Figure 6B shows how there is no release of intracellular calcium when the purified Fc fragment (2 nM) is added. Figure 6C shows how, in order to prove that the fusion protein CCL2-Fc is CCR2 receptor-specific, this receptor is blocked with 5 μg of anti-CCR2 antibody and the subsequent addition of CCL2-Fc (20 nM) does not produce calcium release, indicating that the binding of the fusion protein is carried out specifically by its receptor CCR2.

[0090] Figure 7 shows the inhibitory activity shown by the fusion proteins CCL2-Fc and CCL2 (1+9-76)-Fc in cell migration assays. In the graphs, the migration of the THP1 cells against positive controls (2.5 and 5 nM of MCP1) can be observed and it can also be observed how the incubation of the proteins CCL2-Fc

and CCL2 (1+9-76)-Fc at different concentrations (0.1-12.5 nM) inhibits the migrating response to said stimulus by blocking its specific receptor.

[0091] Figure 8 shows calcium mobilization assays in which intracellular calcium release curves can be observed at different concentrations of both CCL2 (positive control) and of the fusion proteins CCL2-Fc and CCL2(1+9-76)-Fc. These same graphs show that when these proteins are added at concentrations exceeding 3 nM, the addition of CCL2 does not produce calcium mobilization, indicating the desensitization shown by CCR2 against the stimulus produced by its specific ligand. In some assays, SDF, the specific receptor of which is CXCR4, has been added to show that the stimulus caused by the proteins CCL2-Fc and CCL2(1+9-76)-Fc is CCR2-specific and other receptors are not affected in the response to its specific ligand. Likewise, in one of the assays, ionomycin has been added at 10 nM to verify whether the calcium load of the cells used in the assay was optimum.

[0092] Figure 9 shows the result of the IC50 assay of the fusion proteins of the invention. Figure 9A shows the result of the IC50 assay of the fusion proteins CCL2-Fc and CCL2(1+9-76)-Fc. This graph shows the migration of MonoMac cells against CCL2 (positive control) and how the incubation of the cells which will migrate with different concentrations of the fusion proteins CCL2-Fc and CCL2(1+9-76)-Fc (0.06-1 nM) inhibits cell migration to the stimulus produced by CCL2. It can be observed that the IC50 of CCL2-Fc is approximately 0.2 nM and the IC50 of CCL2(1+9-76)-Fc is approximately 0.25 nM. Figure 9B shows the result of the IC50 assay for CCL2-HFc(SER) and CCL2 (1+9-76)-HFc(SER). It can be observed in the graph that the IC50 for any of the two fusion proteins is below 0.06 nM.

[0093] Figure 9C shows the result of the IC50 assay for CXCL12-fcr(-

)and it can be observed in the graph that the IC50 for the fusion protein is below 10 nM. Figure 9D shows the result of the IC50 assay for CCL3fcr(-)and it can be observed in the graph that the IC50 for the fusion protein is above 10 nM.

[0094] Figure 10 shows the sequence of the CCL2 chemokine and the variants thereof: CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A). In the chemokine variant CCL2(1+9-76), from amino acid 2 to 8 of the native chemokine CCL2 have been deleted. In CCL2(P8A), there is a substitution of the proline 8 of the amino acid sequence of the native chemokine CCL2 by alanine. In CCL2(Y13A) there is a substitution of tyrosine 13 of the amino acid sequence of the native chemokine CCL2 by alanine.

[0095] Figure 11 shows the sequence of the CCL5 chemokine and its variant CCL5(S24MP25A). In the chemokine variant there is a substitution of serine 24 of the amino acid sequence of the native chemokine CCL5 by methionine and the proline 25 by alanine.

[0096] Figure 12 describes the sequences of the fusion proteins provided in the Sequence Listing.

Detailed Description of the Invention

[0097] Before the present methods and treatment methodology are described, it is to be understood that this invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0098] As used in this specification and the appended claims, the

singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth in their entirety.

[0100] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference I their entireties.

[0101] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Definitions

[0102] The terms used herein have the meanings recognized and known to those of skill in the art, however, for convenience and completeness, particular terms and their meanings are set forth below.

[0103] If used herein, the terms "Modulation" or "modulates" or "modulating" or "modulator" as applied to a chemokine receptor "modulator" or "modulating" compound or agent, refers to such agent or compound as can up regulate (i.e., activate or stimulate), down regulate (i.e., inhibit or suppress) a response, or that can effect two in combination or apart. Accordingly, a chemokine receptor modulator coupound or agent, would be a compound or agent that modulates at least one biological activity characteristic of or associated with a chemokine. The term "modulating" as related to chemokine receptor binding or signaling, refers to the ability of a compound or agent to exert an effect on leukocyte migration and/or calcium mobilization and signaling (either an increase or decrease in leukocyte migration and/or calcium mobilization and signaling). In the case of a "modulator" that acts as an "inhibitor", one would expect that the inhibitory agent, such as a fusion protein comprising a chemokine variant, if described herein, would compete for binding to the same receptor as the wild type chemokine and would prevent calcium mobilization and signaling normally elicited by binding of the wild type chemokine to its receptor. In the case of a "modulator" that acts as an "enhancer", one would expect that the enhancing agent would retain the binding characteristics of the wild type chemokine and might actually enhance the calcium mobilization and signaling normally observed with the wild type chemokine.

[0104] An "inhibitor", as described herein, is a substance selected from a small organic molecule, or a protein, or peptide, or

nucleic acid molecule, which, in the manner of the present invention, competes for binding of the wild type chemokine to its receptor, but does not induce calcium mobilization and/or signaling. Accordingly, the "inhibitor" may be used to prevent leukocyte migration, which is often seen in inflammatory diseases or conditions. "Inhibitors" may be identified using *in vitro* and *in vivo* assays as described herein. In particular, inhibitors refer to compounds or agents, such as the chemokine fusion proteins as described herein that prevent, decrease or block cell signaling that normally occurs as a result of binding of a wild type chemokine to its receptor.

[0105] A "variant" of a polynucleotide or protein or polypeptide, as the term is used herein, is a polynucleotide or protein or polypeptide that is different from a reference polynucleotide or protein or polypeptide, respectively. Variant polynucleotides are generally limited so that the nucleotide sequence of the reference and the variant are closely related overall and, in many regions, identical. Changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acid sequence encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Alternatively, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a protein or polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions, and truncations in the protein or polypeptide encoded by the reference sequence. Variant proteins or polypeptides are generally limited so that the sequences of the reference and the variant are closely similar overall and, in many regions, identical.

[0106] For example, a variant and reference protein or polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions, and truncations, which may be present or absent in any combination. Such variants can differ in their amino acid composition (*e.g.* as a result of allelic or natural variation in the amino acid sequence, *e.g.* as a result of mutagenesis, *e.g.* as a result of alternative mRNA or pre-mRNA processing, *e.g.* alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation, isoprenylation, lipidation). In the manner of the present application, a chemokine variant retains the ability to bind to the same receptor as the wild type chemokine,. Accordingly, the fusion proteins of the present invention comprise one or more components selected from a chemokine or a chemokine variant and an Fc moiety or a variant thereof from the constant region of an immunoglobulin molecule.

[0107] The variants described in the present invention are noted as follows and the particular changes in the amino acid residues may be found in bold in Figures 10 and 11.

[0108] "CCL2(1+9-76)" refers to a CCL2 variant whereby the amino acid residues at positions 2 through 8 of the native mature CCL2 protein are deleted. This is shown in SEQ ID NO:85. The full length fusion protein containing this CCL2 variant, including the 23 amino acid signal peptide, is found in Seq ID NO:2.

[0109] "CCL2(P8A)" refers to a CCL2 variant that has a change from a proline in the native mature (minus the signal peptide) chemokine at position number 8 to an alanine at position number 8 in the variant CCL2 molecule. This is shown in SEQ ID NO:86. The full length fusion protein containing this CCL2 variant, including the 23 amino acid signal peptide, is found in Seq ID NO: 3.

[0110] "CCL2(Y13A)" refers to a CCL2 variant that has a change from a tyrosine at position 13 in the native mature (minus the signal peptide) chemokine at position number 13 to an alanine at position number 13 in the variant CCL2 molecule. This is shown in SEQ ID NO:87. The full length fusion protein containing this CCL2 variant, including the 23 amino acid signal peptide, is found in Seq ID NO: 4.

[0111] "CCL5-(S24MP25A)" refers to a CCL5 variant that has a change from a serine at position 24 in the native immature (containing the signal peptide) CCL5 chemokine to a methionine at position number 24 in the variant CCL5 molecule and a change from a proline in the native immature (containing the signal peptide) CCL5 chemokine to an alanine in the variant CCL5 molecule at position 25. This is shown in SEQ ID NO:89. The full length fusion protein containing this CCL5 variant, including the 23 amino acid signal peptide, is found in SEQ ID NO: 14.

[0112] "FcR(-)" refers to a modification of the Fc region of an immunoglobulin molecule, which results in lack of binding to an Fc receptor. For example, one such modification is shown in SEQ ID NO: 5, whereby the GluLeuLeu at position 113-115 of SEQ ID NO: 1 is changed to ProValAla.

[0113] The terms "treatment", "treating", and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease, condition, or symptoms thereof, and/or may be therapeutic in terms of a partial or complete cure for a disease or condition and/or adverse effect attributable to the disease or condition.

[0114] "Treatment" as used herein covers any treatment of a disease or condition of a mammal, particularly a human, and includes: (a) preventing the disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet been diagnosed as having it; (b) inhibiting the disease or condition, i.e., arresting its development; or (c) relieving the disease or condition, i.e., causing regression of the disease or condition or amelioration of one or more symptoms of the disease or condition. The population of subjects treated by the method includes a subject suffering from the undesirable condition or disease, as well as subjects at risk for development of the condition or disease.

[0115] As used herein, the terms "fusion protein" and "fusion polypeptide" are used interchangeably and encompass at least a portion of a chemokine, preferably a CC or CXC chemokine, or variant thereof of the present invention, for example, joined *via* a peptide bond to at least a portion of another protein or peptide including but not limited to a portion of the constant region of an immunoglobulin molecule, such as an Fc of an immunoglobulin molecule.

[0116] The term "spacer" or "linker" means one or more molecules, e.g., nucleic acids or amino acids, or non-peptide moieties, such as polyethylene glycol, which may be inserted between one or more component domains. For example, spacer sequences may be used to provide a restriction site between components for ease of manipulation. A spacer may also be provided to enhance expression of the fusion polypeptide from a host cell, to decrease steric hindrance such that the component may assume its optimal tertiary or quaternary structure and/or interact appropriately with its target molecule. For spacers and methods of identifying desirable spacers, see, for example, George et al. (2003) Protein

Engineering 15:871-879, herein specifically incorporated by reference.

CCL3

[0117] Macrophage inflammatory protein 1 alpha (MIP-Ia, CCL3) is a 92 amino acid protein that is constitutively expressed from platelets and bone marrow CD34 progenitor cells. As achemokine, MIP-Ia plays a role in the recruitment of leukocytes to the sites of infection,and is involved in the activation of cells and the inflammatory response. Studies have demonstrated that MIP-Ia is chemotactic for monocytes/macrophages, T cells, neutrophils, eosinophils, basophils, dendritic cells and NK cells. In addition to chemotaxis, an important process in the inflammatory immune response is the migration of cells through the endothelium to tissues sites of inflammation and MIP-Ia also plays a role in the transendothelial migration of monocytes, T lymphocytes, neutrophils, and dendritic cells.

[0118] Additionally, MIP-Ia is involved in the activation of granulocytes, neutrophils, eosinophils, and basophils and it induces the production of proinflammatory cytokines IL-1, IL-6 and TNF from macrophages and fibroblasts. Furthermore, MIP-Ia enhances thesynthesis of interferon (IFN) gamma from activated T cells, which favours a ThI response.MIP-Ia is also crucial in wound repair, as its production by platelets at wound sites recruit and activate macrophages, which in turn phagocytose debris and secrete factors that stimulate wound healing. MIP-Ia also reversibly inhibits the proliferation of hematopoietic stem cells. This has potential clinical utility for patients receiving myelosuppressive therapy, as an inhibitor of progenitor cell proliferation would decrease the toxic effects of this therapy. MIP-Ia may be an effective treatment for the prevention of HIV infection, and additional clinical uses of MIP-Ia would be based on its capacity to recruit and activate leukocytes.

CCL4

[0119] Macrophage inflammatory protein 1 beta (MIP-Ib, chemokine CCL-4) is synthesized as a precursor protein of 92 amino acids and is expressed from activated monocytes, Tlymphocytes, B-lymphocytes, NK cells, dendritic cells and neutrophils. Its expression can be inhibited by IL-IO and IL-4. MIP-Ib exhibits chemotactic properties for the following cell types: monocytes, T cells, neutrophils, eosinophils, immature dendritic cells and NK cells. An important process in an inflammatory immune response is the migration of cells through the endothelium to tissues sites of inflammation and MIP-Ib also plays a role in the transendothelial migration of monocytes, T lymphocytes, neutrophils, and dendritic cells and activation of these cells. Additionally, as MIP-Ib induces the migration and activation of T cells, NK dendritic cells and macrophages it would be an effective antitumour adjuvant inducing the regression of tumours. Furthermore, studies have identified MIP-Ib as one of the major HIV-suppressive factors produced by CD8+ T cells. MIP-Ib may also be used to regulate the febrile response in therapeutic settings as it is capable of antagonising the febrile response induced by MIP-Ia.

CXCL8

[0120] Interleukin-8 (IL-8, CXCL8) is synthesised as a 99 amino acid precursor protein and is induced by a number of different stimuli such as IL-I and TNF. IL-8 promotes the migration of leukocytes to the site of an infection to facilitate an immune response. A number of studies have demonstrated the chemotactic characteristics of IL-8 for a variety of different leukocytes including neutrophils, monocytes, T and B lymphocytes and basophils. IL-8 has also been shown to be involved in the promotion of angiogenesis. Clinically, IL-8 can be used to

mobilize hematopoietic progenitor cells into the peripheral blood for autologous or allogeneic bone marrow transplantation or in the treatment of infertility by promoting follicular maturation and inducing cervical ripening. In certain cancers (e.g. colon and lung cancers) IL-8 may exhibit an anti-tumour effect.

CXCL12

[0121] Stromal cell-derived factor (SDF-1, CXCL12) is a CXC chemokine that selectively activates the CXCR4 chemokine receptor, a chemokine receptor that was first identified in the context of trafficking and homeostasis of immune cells, such as T lymphocytes. It exists in three splice variants, SDF-1α, SDF-1β, SDF-1γ and was originally isolated as a pre-B cell stimulatory factor. Subsequently, it has been determined that CXCR4 regulates several key processes in a wide variety of cancers. Functions of CXCL12 and CXCR4 in cancer first were described in metastatic breast cancer, and more recent studies also have identified roles for this signaling pathway in primary breast tumors.

General Description

[0122] The first aspect of the invention refers to a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin of general formula selected from: (a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$; (b) $Q_A\text{-}Y$; and (c) $Q_A\text{-}(Y)_2$; wherein Q_A is a chemokine or a variant thereof, Q_B is a chemokine or variant thereof different from Q_A and Y is polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0123] A second aspect of the invention refers to a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the

constant region of an immunoglobulin of general formula $(Q_A-Y)_2$ with the condition that: when Q_A is CCL1 from mouse, then Y is not the Fc fragment of the human IgG_1 immunoglobulin; and when Q_A is CCL19 from mouse, then Y is not the Fc fragment of the mouse IgG_{2b} immunoglobulin; wherein Q_A and Y have the previously indicated meanings.

[0124] In the context of the present invention, the term "chemokines" refers to a family of pro-inflammatory signaling molecules involved in the leukocyte activation and migration. Most chemokines have four cysteine residues in highly conserved positions joined to each other by disulphide bridges. The relative position of the first two cysteines in the amino terminus allows its classification in four subfamilies: CC, CXC, CX3C and XC.

[0125] The CC family includes but is not limited to: CCL1 (Genbank, access no. NM_002981), CCL2 (Genbank, access no. NM_002982), CCL3 (Genbank, access no. NM_002983), CCL4 (Genbank, access no. 002984), CCL5 (Genbank, access no. NM_002985), CCL6, CCL7 (Genbank, access no. NM_006273), CCL8 (Genbank, access no. NM_005623), CCL9/CCL10 (Genbank, access no. NP_035468), CCL11 (Genbank, access no. NM_002986), CCL12, CCL13 (Genbank, access no. NM_005408), CCL14 (Genbank, access no. NM_00004166), CCL15 (Genbank, access no. NM_032965), CCL16 (Genbank, access no. NM_004590), CCL17 (Genbank, access no. NM_002987), CCL18 (Genbank, access no. NM_002988), CCL19 (Genbank, access no. NM_006274), CCL20 (Genbank, access no. NM_004591), CCL21 (Genbank, access no. NM_002989), CCL22 (Genbank, access no. NM_002990), CCL23 (Genbank, access no. NM_005064), CCL24 (Genbank, access no. NM_002991), CCL25 (Genbank, access no. NM_005624), CCL26 (Genbank, access no. NM_006072), CCL27 (Genbank, access no. NM_006664), CCL28 (Genbank, access no. AF220210), CCL3L1 (Genbank, access no. NM_021006), CCL3L3 (Genbank, access no. NM_001001437), CCL4L2 (Genbank, access no. NM_207007).

[0126] The CXC subfamily includes but is not limited to: CXCL1 (Genbank, access no. NM_001511), CXCL2 (Genbank, access no. NM_002089), CXCL3 (Genbank, access no. NM_002090), CXCL4 (Genbank, access no. NM_002619), CXCL5 (Genbank, access no. NM_002994), CXCL6 (Genbank, access no. NM_002993), CXCL7 (Genbank, access no. NM_002704), CXCL8 (Genbank, access no. NM_000584), CXCL9 (Genbank, access no. NM_002416), CXCL10 (Genbank, access no. NM_001565), CXCL11 (Genbank, access no. NM_005409), CXCL12 (Genbank, access no. NM_000609/NM_199168), CXCL13 (Genbank, access no. NM_006419), CXCL14 (Genbank, access no. NM_004887), CXCL15(Genbank, access no. AA_H61138), CXCL16 (Genbank, access no. NM_022059).

[0127] The CX3C subfamily includes but is not limited to: CX3CL1 (Genbank, access no. NM_002996).

[0128] Finally, the XC subfamily includes but is not limited to: XCL1 (Genbank, access no. NM_002995) and XCL2 (Genbank, access no. NM_003175).

[0129] A particular embodiment refers to a fusion protein of the invention comprising a chemokine belonging to the CC chemokine subfamily. Said chemokine subfamily is the most numerous and includes, among others, the chemokines CCL2 [MCP-1 (chemoattractant protein for monocytes-1)], CCL5 [RANTES (acronym for *Regulated Upon Activation, Normal T cell Expressed and presumably Secreted)*C], CCL11 (eotaxin-1) and CC13 (MIP-1α). CC chemokines carry out their activity mainly on monocytes and T lymphocytes although they also act on eosinophils, basophils, dendritic cells, B lymphocytes, thymocytes, NK cells, myeloid progenitors and megakaryocytes. Likewise, CC chemokines are produced and secreted to the medium, either in a constitutive manner or in response to different types of stimuli, by several cell types including but not limited to monocytes, macrophages, dendritic cells, eosinophils, NK cells, mastocytes, T lymphocytes, basophils, platelets, hepatocytes, fibroblasts, endothelial cells, keratinocytes and cells from different epithelia.

[0130] A preferred embodiment of the invention refers to a fusion protein comprising a chemokine of the CC subfamily with the ability to interact with the cell surface receptors CCR2 and/or CCR5, which include but are not limited to CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCCL16 and CCL3L1.

[0131] The main ligand of the CCR2 receptor is CCL2 although said recpetor can also bind to other chemokines such the CCL7, CCL8, CCL12, CCL13 and CCL16. CCR2 is expressed in the surface of monocytes, macrophages, B cells and activated T cells. The *in vivo* studies carried out in mice deficient in CCL2 or CCR2 indicate that after different types of treatments, these animals have a defect in the recruitment of monocytes in virtually all tissues (Lu B. et al., 1998). The binding of CCL2 to its receptor gives rise to the formation of a CCL2/CCR2 complex activating a signaling pathway involved in several physiological processes and numerous inflammatory and allergic pathologies, particularly in arthritis, asthma, pulmonary lesion dependent on monocyte-phagocyte, idiopathic pulmonary fibrosis and sarcoidosis, focal ischemia, brain effusion, autoimmune encephalomyelitis, multiple sclerosis, psoriasis, solid tumors, Crohn's disease, ulcerative cholitis, atherosclerosis, obesity, gingivitis and glomerulonephritis. On the other hand, there is data supporting the involvement of CCR2 in the viral pathogenesis of HIV-1, specifically in the entrance of the virus inside the cell.

[0132] The cell receptor CCR5 has the ability to bind to different chemokines, among them CCL5, CCL3, CCL4, CCL8, CCL11, CCL14, CCL16 and CCL3L1. The CCR5 receptor and its ligands are important in viral pathogenesis; for example, CCR5 acts as a co-receptor both for M-trophic strains and for dual T/M-trophic strains of the human immunodeficiency virus (HIV) and there is experimental evidence that its three ligands are potent inhibitors of the entrance of the virus inside the cell. CCR5 is expressed in

the surface of monocytes, mature T cells and immature dendritic cells.

[0133] A more preferred embodiment of the invention refers to a fusion protein comprising a chemokine selected from CCL2 and CCL5.

[0134] The CCL2 chemokine is a protein with an approximate molecular size of 11 kDa secreted by monocytes and other cell types such as the endothelial cells, epithelial cells, fibroblasts, keratinocytes, synovial cells, mesangial cells, osteoblasts, smooth muscle cells, as well as by several tumor cells, among others. An important function of CCL2 is the chemoattraction of monocytes and macrophages from the circulation around the trauma, bacterial infection, exposure to toxin and ischemic sites. CCL2 induces the expression of the integrins required for chemotaxis by acting on monocytes, memory T lymphocytes, basophils and NK cells; it also induces granule release by NK cells and $CD8^+$ T lymphocytes and it is a potent inducer of histamine release by basophils. Studies carried out with mice deficient in CCL2 indicate that this chemokine has a non-redundant role in the regulation of monocyte infiltration during inflammatory processes (Huang et al, 2001).

[0135] Chemokine CCL5 is also a member of the CC chemokine subfamily. It is a protein of approximately 8 kDa secreted by T cells and platelets, furthermore, it also seems to be produced in the epithelium of renal tubules, synovial fibroblasts and several tumor cells. CCL5 is expressed in a constitutive manner and it can be induced by mitogens or antigens in several types of T cells and circulating lymphocytes. *In vitro*, CCL5 is a potent chemoattractant of monocytes, like CCL2, but it is much less effective than the latter. CCL5 also attracts non-stimulated CD4+/CD45RO+ memory T lymphocytes, stimulated CD4+ cells, NK cells, eosinophils, and is the most potent chemoattractant known for $CD8^+$ cells. CCL5 is also a chemoattractant and inducer of histamine release in basophils. Different *in vitro* studies on its

biological activity predict that CCL5 can play an important role in the mediation of inflammatory , viral infection and immune processes

[0136] Chemokines are molecules of an animal origin. A particular embodiment makes reference to a fusion protein of the invention comprising a mammalian chemokine, or a variant thereof; including but not limited to: human, non-human primate, equine, pig, murine, bovine and/or sheep origin. In a preferred embodiment of the invention, said chemokine or variant thereof is of human origin, for example, human CCL2 or human CCL5.

[0137] In the context of the present invention, the variants of a native chemokine are those chemokine molecules having additions, substitutions, deletions or combinations thereof in its amino acid sequence and/or which have been chemically modified with respect to the native chemokine and substantially maintain the ability of said chemokine to bind to its specific receptor. A variant by substitution can have conservative or non-conservative changes. They are conservative changes when the substituted amino acid has a similar structure or similar chemical properties, for example, the change of a leucine for an isoleucine. More rarely, a variant can have non-conservative changes, for example, the change of a glycine for a tryptophan. A chemokine variant will maintain its ability to bind to a specific receptor of at least 10%, 20%, 40%, 60%, although preferably, more than 80%, 90%, 95% or even more preferably, more than 99%, compared to the wild chemokine.

[0138] The ability of a chemokine variant to bind to a specific receptor for said native chemokine can be determined by means of the use of conventional methods known by persons skilled in the art, for example, by measuring any effect associated to the binding of the native chemokine to its specific receptor. As is known, there are different methods belonging to the state of the art such as the calcium release assays (Tsien RY et al., 1982), receptor-ligand binding assays (Seethala R and Fernandes PB,

2001), etc., which allow determining the ability of a chemokine variant to bind to a specific receptor of the native chemokine. Simply by way of illustration, Example 2 describes methods which allow determining the ability of the fusion proteins CCL2-Fc to bind to the CCR2 receptor.

Preparation of Variants of a Native Chemokine

[0139] The variants of a native chemokine can be obtained by several methods known in the state of the art. Said methods include but are not limited to: their isolation from a natural source, in the event that said variations in the amino acid sequences occur naturally, and their obtaining by mutagenesis, which may be oligonucletoide-mediated mutagenesis (or directed mutagenesis), mutagenesis by PCR and cassette mutagenesis of a chemokine variant or non-variant previously prepared.

[0140] In a particular embodiment, the method chosen for preparing chemokine variants having mutations of substitution and/or deletion of one or more amino acids is the oligonucleotide-mediated mutagenesis. This technique is well known in the state of the art (Adehnan JP et al., 1983). In oligonucleotide-mediated mutagenesis, the DNA of a native chemokine is altered by means of the hybridization of an oligonucleotide encoding the desired mutation with a DNA mold. Said DNA mold consists of a single chain DNA of a plasmid or bacteriophage containing the sequence of said native or unaltered chemokine. After hybridization, a DNA polymerase enzyme is used to synthesize the entire second chain complementary of the mold which will thus incorporate the selected mutation in the DNA of the native chemokine. Said oligonucleotides are usually of a length of 25 nucleotides. An optimized oligonucleotide should have between 12 and 15 nucleotides completely complementary to the DNA mold on both sides of the mutation, such that it assures that the oligonucleotide will hybridize to the single chain DNA mold in a suitable manner. The

nucleotides are synthesized using techniques known in the state or the art.

[0141] A particular embodiment of the invention refers to a fusion protein comprising at least one chemokine variant selected from a CCL2 variant and a CCL5 variant substantially retaining the ability thereof to bind to its specific receptors, for example, CCR2 and CCR5 respectively. It has been described that the N-terminal region is critical for the biological activity of chemokines and that therefore, modifications in said region can cause significant changes in the activity of the chemokines in controlling leukocyte traffic. Another particular embodiment refers to a fusion protein of the invention comprising at least, one chemokine variant having modifications in the N-terminal region and substantially retaining the ability to bind to a specific receptor thereof. By way of a non-limiting illustration, said modifications comprise the deletion of amino acids in the N-terminal region of the native chemokine or alternatively, the substitution of one or more amino acids in the N-terminal region of said native chemokine.

[0142] A particular preferred embodiment refers to a fusion protein comprising at least one chemokine variant selected from a CCL2 variant and a CCL5 variant, having modifications in the N-terminal region and substantially retaining the ability to bind to its specific receptor, for example: CCR2 and CCR5, respectively. By way of a non-limiting illustration, said chemokine variant is a CCL2 variant in which the amino acid 2 to the amino acid 8 have been deleted from the native chemokine CCL2, or a CCL2 variant modified by substitution of one or more amino acids of the N-terminal region, or a CCL5 variant modified by substitution of one or more amino acids of the N-terminal region. In a more preferred particular embodiment, said CCL2 or CCL5 variant having modifications in the N-terminal region and substantially retaining the ability to bind to a specific receptor thereof is selected

from CCL5(S24MP25A), CCL2(1+9-76), CCL2(P8A) AND CCL2(Y13A) (See example 1.1.4).

[0143] A particular preferred embodiment refers to a fusion protein comprising at least one chemokine variant selected from a CCL2 variant and a CCL5 variant, having modifications in the N-terminal region and substantially retaining the ability to bind to its specific receptor, for example: CCR2 and CCR5, respectively. In a more preferred particular embodiment, said CCL2 or CCL5 variant having modifications in the N-terminal region and substantially retaining the ability to bind to a specific receptor thereof is selected from CCL5(S24MP25A), CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A) (See example 1.1.4).

[0144] Another member of the fusion protein is the polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin. In a particular embodiment, the fusion protein of the invention is a protein in which the polypeptide Y comprises, or is formed by an optionally modified portion of the constant region of an immunoglobulin.

[0145] Immunoglobulins (Ig) are formed by four chains, two heavy chains and two light chains, joined to each other by means of disulphide bridges. Immunoglobulins can be of different isotypes: IgG, IgA, IgM, IgD or IgE. Depending on the Ig isotype, the constant region of the heavy chain is formed by 3 or 4 domains, denominated domains of the constant region of the heavy chain (e.g.: CH1, CH2, CH3, CH4). Some isotypes further contain a region denominated hinge, located between the domains CH1 and CH2.

[0146] In a particular embodiment, the fusion protein of the invention is a protein in which the polypeptide Y comprises, or is formed by a portion of the constant region of a molecule of mammalian immunoglobulin, including but not limited to: human, non-human primate, equine, pig, murine, bovine and/or sheep origin. In the cases in which the portion of the constant region of immunoglobulin is not of a human origin, said portion of

immunoglobulin can be humanized. (Jones PT et al., 1986). In a preferred embodiment of the invention, the portion of the constant region of immunoglobulin of the fusion proteins of the invention is of human origin.

[0147] In the context of the present invention, a portion of the constant region of an immunoglobulin refers to the constant region of the complete heavy chain, a fragment, homologous sequence and/or variant thereof; as well to the constant region of the complete light chain, a fragment, homologous sequence and/or variant thereof.

[0148] An average person skilled in the art will understand that the term "portion of the constant region of immunoglobulin" includes fragments, homologous sequences and variants thereof, including those constant regions of immunoglobulins which have been chemically modified, for example by means of pegylation (Aslam and Dent, 1998).

[0149] In the context of the present invention, homologous molecules are understood to be those molecules the amino acid sequence of which has a high degree of similarity, of at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% with respect to a determined sequence. Said sequences can be, for example, variants derived from several species, or derived from said sequence by modification by means of truncations, deletions, modification or addition of amino acids. The percentage of identity between two amino acid sequences is determined by means of standard sequence alignment algorithms known in the state of the art, such as for example BLAST, described by Altschul SF et al, 1990.

[0150] In a preferred embodiment of the invention, the polypeptide Y of the fusion protein of the invention comprises at least one domain of the constant region of the heavy chain of an immunoglobulin. The constant region of the heavy chain of an immunoglobulin molecule comprises a CH1 domain, CH2 domain, a CH3

domain and in some immunoglobulin isotypes, also a CH4 domain. In a more preferred embodiment, the polypeptide Y of the fusion protein of the invention comprises at least one domain of the heavy chain of an immunoglobulin selected from the CH2 domain and the CH3 domain of the heavy chain of an immunoglobulin.

[0151] In a still more preferred embodiment, the polypeptide Y of the fusion protein of the invention comprises, or is formed by the CH2 and CH3 domains of the heavy chain of an immunoglobulin. In a specific embodiment, an Fc fragment comprising the CH2 and CH3 domains of the heavy chain of an immunoglobulin, can also include the hinge region of an immunoglobulin molecule or a fragment thereof. Depending on the immunoglobulin isotype, IgG, IgM or IgE, among others, the Fc fragment significantly increases the average half-life of the fusion protein with respect to the original peptide molecule and it provides it with effector functions. In those cases in which the hinge region is maintained, the latter provides flexibility to the fusion protein such that the Fc fragment and the non-immunoglobulin domain can work independently.

[0152] An Fc fragment is synthesized in a monomeric form and is secreted extracellularly as a dimer. In the context of the present invention, the monomer form of the Fc fragment is denominated HFc. The Fc fragment can be an Fc fragment of an IgG1, IgG2, IgG3 and/or IgG4 or a hybrid Fc fragment, i.e., formed by antibody domains of different isotypes, for example, an Fc fragment comprising a CH2 and CH3 region of IgG2, and the hinge region derives from IgG1. In a still more preferred embodiment of the invention, the portion of a constant region of immunoglobulin consists of an Fc fragment of an IgG1 immunoglobulin, a fragment or variant thereof.

[0153] From a therapeutic point of view, it is interesting to have proteins which can be easily purified, highly soluble and with a long half-life in order to manufacture therapeutically

effective drugs suitable for clinical treatment. The use of an Fc fragment of an immunoglobulin fused to a protein has shown that it increases the production and secretion thereof, as well as that it facilitates its purification. The Fc fragment, as a very soluble and relatively large protein carrier, can potentially extend the circulating half-life of pharmacological proteins *in vivo*, protecting them from degradation and allowing a slower biological clearance, which would allow reducing the number of administrations of the therapeutic molecule.

[0154] In a preferred embodiment, the fusion protein of the invention is a homodimer of general formula $(Q_A\text{-}Y)_2$ wherein Q_A is selected from CCL2, CCL5 or a variant thereof, and the polypeptide Y comprises a native Fc fragment.

[0155] In another particular embodiment, the polypeptide Y comprises a variant of a portion of the constant region of an immunoglobulin molecule, for example an Fc variant of an IgG immunoglobulin. In the context of the present invention, an Fc variant refers to a modified molecule from a native Fc molecule but which still conserves a binding site for the corresponding wild Fc receptor FcRn. In the context of the present invention, a native Fc refers to an Fc which has not been modified by man. Patent application WO 97/34631 describes examples of Fc variants, as well as interactions with the wild receptor FcRn. The Fc region can be modified using widely known methods such as directed mutagenesis, with the aim of providing a modified IgG molecule, a fragment or portion thereof that binds to the FcRn receptor. Such modifications may include changes in regions far-away from the site of binding or contact with FcRn as well as modifications in the binding site preserving or increasing the binding to the receptor.

[0156] A native Fc comprises residues which can be modified or eliminated because they provide the Fc fragment with structural characteristics or biological activities that are not essential

for the fusion proteins of the present invention. Thus, in the context of the present invention, an Fc variant includes but is not limited to an Fc molecule lacking one or more of the sites or residues affecting or involved in: (1) formation of disulphide bridges, (2) incompatibility with a specific host cell, (3) N-terminal heterogenicity since it is expressed in a specific host cell, (4) glycosilation, (5) interaction with the complement, (6) binding to an Fc receptor different from the wild Fc receptor, or (7) cell toxicity mediated by antibodies (ADCC).

[0157] In a particular embodiment of the invention, the constant region of an immunoglobulin molecule consists of an Fc variant which has been modified such that it prevents the binding thereof to its specific receptor, thus avoiding the effector actions mediated by the Fc fraction of immunoglobulin, such as for example, the fixing to the complement. The use of Fc fragments could produce a non-desired phenomenon because the Fc fragment by itself can be recognized by the Fc receptors of the immune system, causing cell activation responses associated to the immune effector functions of the Fc and reducing the circulating half-life of the therapeutic protein. This can be prevented by means of directed mutagenesis to eliminate the residues of the Fc domain involved in the binding to its receptor. In a particular preferred embodiment, said Fc variant is a variant of a human IgG1 immunoglobulin having a substitution mutation of one or more amino acids, selected from E233P, L234V and L235A.

[0158] In another particular embodiment, the fusion protein of the invention is a monomer of general formula Q_A-Y, where Q_A is a chemokine or a variant thereof and the polypeptide Y comprises an Fc variant lacking or having modifications in the residues involved in the formation of disulphide bridges necessary for forming intercatenary dimers and therefore, it prevents the dimerization of Fc monomers (HFc). In a preferred embodiment, said Fc variant is an Fc variant of a human IgG1 immunoglobulin having

a mutation consisting of replacing the amino acids C226 and/or C229 with Serine and/or Alanine by means of substitution. In another particular embodiment, the fusion prctein of the invention is a monomer of general formula Q_A-Y, where Q_A is a chemokine or a variant thereof and the polypeptide Y comprises an Fc variant lacking or having modifications in the residues involved in the formation of disulphide bridges. In another particular preferred embodiment, the fusion protein of the invention is a momomer of general formula Q_A-Y, where Q_A is selected from CCL2, CCL5 and a variant thereof and the polypeptide Y comprises an Fc variant lacking or having modifications in the residues involved in the formation of disulphide bridges. In a specific embodiment, said Fc variant is an Fc variant of a human IgG1 immunoglobulin.

[0159] In another particular embodiment of the invention, the constant region of an immunoglobulin molecule present in the polypeptide Y consists of an Fc variant in a human IgG1 immunoglobulin which has been modified such that it favors the formation of heterodimers. In a preferred embodiment, the constant region of an immunoglobulin molecule consists of an Fc variant in which the CH3 domain of Fc has been redesigned to favor the heterodimerization between chains over homodimerization. This concept was first developed by Rigdway et al., 1996 and is based on the idea that mutations can be introduced in one of the halves of the CH3 dimer, such that the steric complement required for the CH3/CH3 association forces the mutated CH3 domain to pair with another CH3 domain having different mutations which accommodate. Specifically, a knob mutation carried out in one of the chains in order to introduce a larger residue in the CH3 dimer interface and create a steric barrier for the homodimerization. In order to promote the heterodimerization, a complementary mutation forming a hole is produced by means of genetic engineering techniques in the CH3 domain of the other Fc chain. The co-expression of these two Fc chains gives rise to the formation of a majority of the

heterodimeric product. In a more preferred embodiment, said "knob into hole" modification comprises mutations in the knob chain selected from Y349C and T366W and mutations in the hole chain selected from D356C, T366S, L368A and Y407V, as described by Merchant M et al., 1998.

[0160] In another preferred embodiment, the fusion protein of the invention is a heterodimer of general formula $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$, where Q_A is CCL2 or a variant thereof and Q_B is a CCL5 or a variant thereof and the polypeptide Y comprises an Fc variant which has been modified such that it favors the formation of heterodimers.

[0161] In another preferred embodiment, the fusion protein of the invention is a monofunctional dimer of general formula $Q_A\text{-}Y_2$, where Q_A is CCL2, CCL5 or a variant thereof and the polypeptide Y comprises an Fc variant which has been modified such that it favors the formation of heterodimers.

[0162] A fusion protein of the invention can further comprise other elements such as a spacer peptide or linker, a tag peptide, a signal peptide, among others.

[0163] A particular embodiment refers to a fusion protein of the invention further comprising a spacer peptide between said chemokine or variant thereof and said polypeptide Y. The presence of a linker peptide, preferably a flexible linker, between both, increases the flexibility of the chemokine domains and provides resistance to degradation. In a preferred embodiment, said spacer peptide is a flexible peptide linker with a length of 20 or less amino acids. In a more preferred embodiment, the peptide linker comprises 2 or more amino acids selected from the group consisting of glycine, serine, alanine and threonine, for example, it comprises the glycine and serine amino acid sequence GGGGS. In another preferred embodiment, said spacer peptide comprises the entire or a portion of the hinge region of an immunoglobulin.

[0164] Another particular embodiment refers to a fusion protein of the invention further comprising a tag peptide. Said tag

peptide includes but is not limited to: polyhistidine tags (His-tags) (for example H_6 and H_{10}, etc. or other tags for use in IMAC systems, for example, Ni^{2+} affinity columns, etc.), GST fusions, MBP fusions, streptavidine-tags, the BSP biotinylation target sequence of the bacterial enzyme BIRA and tag epitopes that are directed by antibodies (for example c-myc tags, flag-tags, among others). As will be observed by a person skilled in the art, said tag peptide can be used for purification, inspection, selection and/or visualization of the fusion protein of the invention.

[0165] Another particular embodiment refers to a fusion protein of the invention further comprising a signal peptide or leader peptide directing the fusion protein of the invention to the extracellular medium. In the present invention, for the secretion of the fusion protein of the invention from the host cell to the exterior of the cell, a signal peptide codified in the sequence of a native chemokine can be used or alternatively, a DNA encoding a heterologous signal sequence can be used, for example a signal peptide of another chemokine or of a secreted immunoglobulin molecule.

[0166] In another aspect, the invention refers to a nucleic acid, hereinafter nucleic acid of the invention, encoding a fusion protein of the invention comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin of general formula selected from: (a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$; (b) $Q_A\text{-}Y$; and (c) $Q_A\text{-}(Y)_2$; where Q_A is a chemokine or a variant thereof, Q_B is a chemokine or a variant thereof different from Q_A and Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0167] The nucleic acid can also contain, if desired, a nucleotide sequence encoding a spacer peptide or linker, a nucleotide sequence encoding a tag peptide, a nucleotide sequence encoding a signal sequence or peptide, etc.

[0168] The nucleic acid of the invention encoding a fusion protein of the invention can encode in the direction 5' to 3': (1) a chemokine or variant thereof and a constant region of immunoglobulin; (2) a signal sequence, a constant region of immunoglobulin and a chemokine or variant thereof, (3) a signal sequence, a chemokine or variant thereof and a constant region of immunoglobulin. In a preferred embodiment, the nucleic acid molecule encodes in the direction 5′ to 3′: a chemokine or variant thereof comprising in its sequence a signal peptide and a constant region of immunoglobulin, i.e. said polypeptide Y is bound to the carboxyl terminal end of Q_A and/or Q_B.

Preparation of Fusion Proteins

[0169] The fusion proteins of the invention can be obtained by several methods such as recombinant DNA techniques, covalent or non-covalent binding of the components of the fusion protein, among others. Example 1 describes the obtaining of fusion proteins of the invention in a non-limiting manner.

Cell Transfection

[0170] In a particular embodiment, the nucleic acid of the invention is transfected in a host cell using recombinant DNA techniques. The fusion proteins of the invention can be produced by using recombinant expression vectors known in the state of the art. The term expression vector refers to a replicative DNA construct used to express DNA encoding the fusion protein of the invention and including a transcriptional unit comprising an assembly of (1) genetic element/s having a regulating role in gene expression, for example, promoters, operators or enhancers, bound operatively to (2) a DNA sequence encoding a fusion protein of the invention which is transcribed to messenger RNA and translated to protein and (3) suitable sequences for the initiation and termination of the transcription and translation. The selection of a promoter and other regulating element/s generally varies according to the host cell used. A preferred expression vector of

the invention is an expression vector derived from pcDNA3 (Invitrogen, Carlsbad, CA; USA).

[0171] Suitable host cells include: prokaryotic cells, yeasts or eukaryotic cells. Preferred host cells are eukaryotic cells. Several insect or mammalian cell culture systems can be used to express the recombinant protein. Baculovirus systems for the production of proteins in insect cells are well known in the state of the art. Likewise, some examples of suitable cell lines of host mammals include but are not limited to: NS/0 cells, L cells, C127, 3T3, Chinese hamster ovary cells (CHO), human embryonic kidney cells (HEK-293), HeLa and BHK; and CV-1 cells (ATCC CCL70) and COS-7 cells, both derived from monkey kidney.

Expression Vectors

[0172] The expression vectors of mammals can comprise non-transcribable elements as a replication origin, a suitable promoter and an enhancer bound to the gene to be expressed and other non-transcribable 5' or 3' flanking sequences and non-translatable 5' or 3' sequences as the necessary ribosome binding sites, polyadenylation sites, splicing acceptor and donor sites and transcription termination sequences. The promoters and enhancers most commonly used are derivatives of polyoma, adenovirus-2, simian virus 40 (SV-40) and human cytomegalovirus (CMV).

[0173] The present invention also provides a process for obtaining the fusion proteins of the present invention comprising the culture of a host cell transformed with an expression vector comprising a nucleic acid sequence of the invention encoding the fusion protein of the invention under conditions promoting the expression thereof. The fusion protein of the invention can be purified from the culture medium or from cell extracts. For example, from supernatants of the expression systems secreting the recombinant protein in the culture medium. The fusion protein of the invention is conveniently captured using a suitable purification matrix, for example a G protein-coupled matrix

(Akerstrom B et al, 1985), being, once eluted, concentrated using a commercially available protein concentrating filter, for example, Amicon or Millipore Pellicon, as known in the state of the art.

[0174] In another aspect, the present invention refers to a process for obtaining a fusion protein with an activity inhibiting the binding of a chemokine to a specific receptor thereof in vitro, comprising the following steps:

- generating a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin;
- testing the ability of said fusion protein to inhibit the binding of said chemokine to a specific receptor thereof; and
- selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof in vitro.

[0175] Said fusion protein can be obtained by conventional methods, as mentioned previously. In a particular embodiment, said fusion protein is a fusion protein of the invention, which can be obtained as mentioned previously. The ability of said fusion protein to inhibit the binding of said chemokine to a specific receptor thereof can be determined by conventional methods, as mentioned previously. Finally the fusion proteins with an activity inhibiting the binding of said chemokine to a specific receptor thereof *in* vitro are selected.

[0176] In another aspect, the present invention refers to a process for selecting a fusion protein having an activity inhibiting the binding of a native chemokine to a specific receptor thereof *in* vitro, improved with respect to a variant of said native chemokine, comprising the following steps:

- generating a fusion protein comprising (i) a variant of a native chemokine and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin;
- testing the ability of said fusion protein to inhibit the

binding of said native chemokine to a specific receptor thereof; and

- selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro* greater than said chemokine variant.

[0177] Said fusion protein can be obtained by conventional methods, as mentioned previously. In a particular embodiment, said fusion protein is a particular case of the fusion proteins of the invention, in which Q_A is a variant of a native chemokine which can be obtained as described previously. The ability of said fusion protein to inhibit the binding of said chemokine to a specific receptor thereof can be determined by conventional methods, as described previously. Finally, the fusion proteins having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro* greater than said chemokine variant are selected.

Pharmaceutical compositions and medical uses

[0178] The fusion proteins provided by this invention can be used in several applications, including the diagnosis and therapeutic research and applications.

[0179] Chemokines carry out their role by means of the activation of specific receptors. However, the chemokine system has a certain promiscuity in the use of its receptors, in the sense that there are chemokines capable of binding to more than one receptor and receptors having more than one chemokine ligand. In this way, depending on the chemokine forming part of the fusion protein of the invention, said fusion protein can bind to different chemokine receptors. In this way, the fusion protein of the invention will be useful in the treatment of a series of determined pathologies depending on the chemokine receptor involved. In addition to its role in leukocyte recruitment, it is currently known that chemokines carry out important functions in cell proliferation and apoptosis, tissue morphogenesis, hematopoiesis, as well as in

angiogenesis and in the development of specific immune responses. Likewise, the involvement of chemokines and their receptors in autoimmune diseases and in protozoal (eg. infections caused by *Plasmodium vivax*) and viral infections (e.g. infections caused by the human immunodeficiency virus (HIV), etc.) have also been described.

[0180] Therefore, the fusion proteins provided by this invention can be used in the treatment of several diseases and pathologies, for example, inflammatory, tumor, autoimmune, protozoal, viral and bacterial diseases.

[0181] Examples of symptoms or pathologies that can be treated by means of the use of a fusion protein of the invention include: pain, acute inflammation, chronic inflammation, rheumatoid arthritis, psoriasis, atopic dermatitis, asthma, chronic obstructive pulmonary disease (COPD), respiratory disease, arthritis, inflammatory disease of the intestine such as Crohn's disease and ulcerative cholitis, septic shock, endotoxic shock, sepsis due to gram negative bacteria, toxic shock syndrome, brain ictus, ischemic damage due to reperfusion, renal disease due to reperfusion, glomerulonephritis, thrombosis, Alzheimer's disease, graft versus host disease, allogenic transplant rejection, malaria, acute respiratory distress syndrome, retarded hypersensitivity reaction, atherosclerosis, cerebral ischemia, cardial ischemia, osteoarthritis, multiple sclerosis, restenosis, angiogenesis, osteoporosis, gingivitis, infections caused by respiratory viruses, herpesvirus, hepatitis virus, HIV, virus associated to Kaposi's sarcoma, meningitis, cystic fibrosis, labour, cough, itching, multiorgan failure, trauma, strains, sprains, bruises, psoriatic arthritis, encephalitis, central nervous system (CNS) vascultis, traumatic brain damage, CNS tumors, subarachnoid hemorrhage, postsurgical trauma, interstitial pneumonitis, hypersensitivity, arthritis induced by crystals (hydroxyapatite, uric acid, etc.), acute pancreatitis, chronic

pancreatitis, acute alcoholic hepatitis, necrosing enterocolitis, chronic sinusitis, angiogenic ocular disease, ocular inflammation, retinopathy of prematurity, diabetic retinopathy, macular degeneration, corneal neovascularization, polymyositis, vasculitis, acne, gastric ulcer, duodenal ulcer, celiac disease, oesophagitis, glossitis, respiratory tract obstruction, bronchial hyperreactivity, bronchiectasis, bronchiolitis, bronchiolitis obliterans, chronic bronchiolitis, cor pulmonale, dyspnea, emphysema, hypercapnia, hyperinsufflation, hypoxemia, inflammation induced by hyperoxia, hypoxia, pulmonary volume reduction due to surgical intervention, pulmonary fibrosis, pulmonary hypertension, right ventricular hypertrophy, peritonitis associated with continuous ambulatory peritoneal dialysis (CAPD), granulocytic ehrlichiosis, sarcoidosis, small respiratory tract disease, ventilation-perfusion alteration, wheezing, colds, gout, alcoholic hepatic disease, lupus, burn therapy, periodontitis, cancer, damage due to transplant by reperfusion or acute transplant rejection.

[0182] In a particular embodiment, the fusion proteins provided by this invention can be used in the treatment of inflammatory, tumorous, autoimmune, protozoal, viral and/or bacterial diseases.

[0183] Therefore, in another aspect, the invention relates to a pharmaceutical composition, hereinafter denominated "pharmaceutical composition of the invention" comprising a fusion protein provided by this invention together with, optionally, a pharmaceutically acceptable carrier. In the sense used in this description, the expression "fusion protein provided by this invention" refers to a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of the general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) Q_A-$(Y)_2$; or

(d) $(Q_A$-$Y)_2$

where

Q_A is a chemokine or a variant thereof;

Q_B is a chemokine or a variant thereof, different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

[0184] The pharmaceutical composition of the invention can be administered alone or in combination with one or more pharmaceutically acceptable carriers in single or multiple doses. Pharmaceutically acceptable carriers include inert solid diluents or filler materials, sterile aquous solutions and several organic solvents. In a particular embodiment, the pharmaceutical composition of the invention is prepared in the form of an aqueous solution or suspension in a pharmaceutically acceptable carrier such as a saline solution, a phosphate buffered saline solution (PBS), or any other pharmaceutically acceptable carrier. Illustrative and non-limiting examples of pharmaceutically acceptable carriers for the administration of a fusion protein of the invention include, for example, a sterile saline solution (for example, 0.9% NaCl). The pharmaceutical composition of the invention can also contain, if necessary, other auxiliary substances or pharmaceutically acceptable carriers, such as co-solvents, additives, e.g. pharmaceutically acceptable preservatives, acids, bases or pharmaceutically acceptable buffers for adjusting the pH, surfactants, etc. Likewise, it is possible to add metal chelating agents to stabilize the suspension.

[0185] The pharmaceutical composition of the invention comprises a therapeutically effective amount of a fusion protein provided by this invention. In the sense used in this description, the expression "therapeutically effective amount" refers to the amount of fusion protein provided by this invention contained in the pharmaceutical composition of the invention calculated to produce

the desired effect and it will be generally determined, among other causes, by the characteristics of the fusion protein of the invention and the effect to be achieved. Generally, the therapeutically effective amount of fusion protein provided by this invention to be administered will depend, among other factors, on the fusion protein provided by this invention to be administered, on the subject to be treated, on the pathology he/she suffers from, on its severity, on the chosen administration form, etc. Due to this reason, the doses mentioned in this invention must only be considered as guidelines for the person skilled in the art, and the latter must adjust the doses according to the variables cited previously.

[0186] The therapeutic dose will generally range from approximately 0.001 to 100 mg/kg of the body weight of the subject to be treated. Nevertheless, there may be variations depending on the response of the individual subject to said drug, as well as the type of the pharmaceutical formulation chosen and the time period and interval in which such administration is carried out. The fusion protein dose can be repeated, depending on the condition of the patient and his/her evolution, at time intervals (days, weeks or months) which will have to be established in each case by the specialist.

[0187] For its administration to a subject in need of treatment, the pharmaceutical composition of the invention can be administered by any suitable route, for example, by an oral (e.g., oral, sublingual, etc.), parenteral (e.g., subcutaneous, intramuscular, intravenous, intra-articular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracerebellar, intracerebroventricular, intracolon, intracervical, intragastric, intrahepatic, intramyocardial, intra-osseal, intrapelvic, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intra-rectal, intra-renal, intra-retinal, intraspinal, intrasynovial, intrathoracic, intrauterine,

intravesicular, etc.), vaginal, rectal, urethral, intranasal (pulmonary), topical, subcutaneous (e.g., transdermal, etc.), ophthalmic, auricular route etc., preferably parenterally.

[0188] The preferred administration route is the parenteral route (e.g. intravenous, intramuscular, subcutaneous or intramedullary route).

[0189] The pharmaceutical composition of the invention formed by combining the fusion protein provided by this invention and the pharmaceutically acceptable carrier or carriers can be administered in a plurality of solid, liquid dosage forms etc. such as tablets, capsules, syrups, solutions, suspensions for oral use, injectable solutions or suspensions, aerosols, drops, gels etc. If desired, these pharmaceutical compositions can contain additional ingredients such as flavoring agents, binders, excipients and the like. In this way, the pharmaceutical composition of the invention will incorporate the suitable pharmaceutically acceptable carriers, excipients and suitable pharmaceutically acceptable auxiliary substances according to the selected pharmaceutical form of administration. Said pharmaceutical forms of administration can be prepared by conventional methods. A review of the different pharmaceutical forms of drug administration and their preparation can be found in "Tratado de Farmacia Galénica", by C. Faulí i Trillo, 10th Edition, 1993, Luzán 5, S.A. de Ediciones. In any case, the pharmaceutical composition of the invention can be administered using the suitable equipment, apparatus and devices known by the persons skilled in the art, for example, catheters, cannulas, needles etc.

[0190] In a particular embodiment, for its parenteral administration, solutions of the fusion protein provided by this invention can be used in an oil solution, for example, in sesame or peanut oil, etc., in an aqueous propylene glycol solution, in a sterile aqueous solution etc. If necessary, such aqueous solutions will have been suitably buffered, and the liquid diluent should first be made

isotonic with a sufficient amount of glucose or saline solution. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. All the sterile aqueous media used are easily available by means of conventional methods known by persons skilled in the art.

[0191] The pharmaceutical composition of the invention can also be used together with other additional drugs in order to provide a combination therapy. In a particular embodiment, said additional drugs are useful drugs in the prevention and/or treatment of inflammatory, tumor, autoimmune or viral pathologies in active form. Illustrative, non-limiting examples of additional drugs which can be used to provide a combination therapy include: an anti-neoplastic agent, a TNF antagonist, a muscular relaxant, an anti-inflammatory agent, an anti-viral agent, an analgesic, an anesthetic, a sedative, a neuromuscular blocking agent, an anti-microbial agent, a corticosteroid, an anabolic steroid, a vaccine, an immunosupressor, an anti-angiogenic agent, a hormone, an antidepressant drug, an antipsychotic drug, a stimulant, an asthmatic medication, a beta-agonist, a cytokine or a cytokine antagonist.

[0192] Said additional drugs can form part of the same pharmaceutical composition provided by this invention or, alternatively, they can be provided in the form of a separate composition for its simultaneous or sequential administration to the pharmaceutical composition of the invention. The combined administration therefore includes the co-administration, using separate compositions or a single composition, and the consecutive or sequential administration in any order, there being a time period in which preferably both (or all) the active ingredients carry out their biological functions simultaneously.

[0193] In a particular embodiment, the fusion protein provided by this invention can be used in the manufacture of an anti-

inflammatory, antitumor or antiviral composition. In a more particular embodiment, the fusion protein of the invention is used in the manufacture of an antiviral composition. In an even more particular embodiment, the fusion protein of the invention is used in the manufacture of a pharmaceutical composition for the treatment of viral infections, for example, infections caused by respiratory virus, herpes virus, hepatitis virus, human immunodeficiency virus (HIV), virus associated to Kaposi's sarcoma, etc. In a specific embodiment, the fusion protein of the invention is used in the manufacture of a pharmaceutical composition for the treatment of the infection caused by HIV-1.

[0194] In another particular embodiment, the fusion protein provided by this invention is used in the manufacture of a pharmaceutical composition for the treatment of inflammatory or autoimmune diseases in which the receptors or ligands are involved in processes among which are: pain, acute inflammation, chronic inflammation, rheumatoid arthritis, psoriasis, atopic dermatitis, asthma, chronic obstructive pulmonary disease (COPD), respiratory disease, arthritis, inflammatory diseases of the intestine such as Crohn's disease and ulcerative cholitis, septic shock, endotoxic shock, sepsis due to gram negative bacteria, toxic shock syndrome, brain ictus, ischemic damage due to reperfusion, glomerulonephritis, thrombosis, Alzheimer's disease, graft versus host disease, allogenic transplant rejection, malaria, acute respiratory distress syndrome, retarded hypersensitivity reaction, atherosclerosis, cerebral ischemia, cardiac ischemia, osteoarthritis, multiple sclerosis, restenosis, angiogenesis, osteoporosis, gingivitis, meningitis, cystic fibrosis, labour, cough, itching, multiorgan failure, trauma, strains, sprains, bruises, psoriatic arthritis, encephalitis, central nervous system (CNS) vasculitis, traumatic brain damage, CNS tumors, subarachnoid hemorrhage, postsurgical trauma, interstitial pneumonitis, hypersensitivity, arthritis induced by crystals (hydroxyapatite,

uric acid, etc.), acute pancreatitis, chronic pancreatitis, acute alcoholic hepatitis, necrosing enterocolitis, chronic sinusitis, angiogenic ocular disease, ocular inflammation, retinopathy of prematurity, diabetic retinopathy, macular degeneration, corneal neovascularization, polymyositis, vasculitis, acne, gastric ulcer, duodenal ulcer, celiac disease, oesophagitis, glossitis, respiratory tract obstruction, bronchial hyperreactivity, bronchiectasis, bronchiolitis, bronchiolitis obliterans, chronic bronchiolitis, cor pulmonale, dyspnea, emphysema, hypercapnia, hyperinsufflation, hypoxemia, inflammation induced by hyperoxia, hypoxia, pulmonary volume reduction due to surgical intervention, pulmonary fibrosis, pulmonary hypertension, right ventricular hypertrophy, peritonitis associated with continuous ambulatory peritoneal dialysis (CAPD), granulocytic ehrlichiosis, sarcoidosis, small respiratory tract disease, ventilation-perfusion alteration, wheezing, colds, gout, alcoholic hepatic disease, lupus, burn therapy, periodontitis, cancer, damage due to transplant by reperfusion or acute transplant rejection. Illustrative and non-limiting examples of cancers which can be potentially treated with the fusion proteins provided by this invention include breast cancer, melanoma, ovarian cancer, pancreatic cancer as well as metastasis in different organs, for example, liver, lung, bone, etc.

Diagnostic Applications

[0195] In a particular embodiment, the fusion protein provided by this invention is used in the manufacture of a composition for the diagnosis in vitro or *in vivo* of diseases in which the levels of a certain chemokine are altered. The detection *in vivo* of chemokine receptors can be carried out by means of molecular image techniques. The suitable labeled fusion proteins provided by this invention form specific contrasts and can be detected according to the presence of binding to a specific chemokine receptor thereof. In a more particular embodiment, the fusion protein of the

invention is labeled with a suitable marker, such as a fluorescent, chemoluminiscent, isotope marker etc. In a particular embodiment, the fusion proteins provided by this invention can be used to specifically detect the presence of chemokine receptors , for example, in T cells, monocytes and other cells involved in any of the processes described previously. Molecular image techniques (e.g. among which are nuclear magnetic resonance (NMR), positron emission tomography (PET), computed axial tomography (CAT), high resolution X-rays, and signal emission methods (fluorescent, (bio)luminescent signals, etc.) allow carrying out specific detection studies *in vivo,* such that in animal experimentation, they allow studying the evolution of a pathology or the effect of a drug in the animal itself without having to sacrifice it. Likewise, in humans, it allows the pharmacological study or the study of the pathology in different tissues in a non-invasive and virtually painless manner.

[0196] In another aspect, the invention relates to the use of a fusion protein provided by this invention in the qualitative or quantitative detection of the presence of chemokine receptors in the surface of a cell *in vitro.* Such fusion proteins may be used in similar methods to those already known and used for antibody labelling of cells and tissues. Said detection can be carried out, for example, by means of a process comprising:

a. providing at least one cell expressing the receptor corresponding to the fusion protein provided by this invention;

b. incubating said fusion protein in saturating conditions until equilibrium is reached; and

c. determining the amount of said protein bound to the cells.

[0197] In order to detect and/or quantify the presence of chemokine receptors in the cell surface, a composition comprising a cell or cell fraction (for example, cell membrane fraction) is put into contact with the fusion protein provided by this invention, which is bound to the corresponding specific receptor or a portion

thereof under conditions suitable for said binding, such that said binding can be monitored. The detection of the fusion protein, indicative of the formation of a complex between the corresponding receptor and the fusion protein provided by this invention, indicates the presence of said receptor. The binding of the fusion protein to the cell can be determined, for example, by means of affinity assays or binding assays. In this way, the method of the invention can be used to detect the expression of a specific chemokine receptor in the cells of a subject (for example, in a sample, such as a body fluid, like blood, saliva or another available sample). The expression levels of the corresponding receptor in the cell surface of the immune system, such as T cells or monocytes, can also be determined by conventional methods, for example, by flow cytometry, such that the expression level (labeling intensity) can be correlated with the susceptibility, progress or risk of suffering from a disease in which the expression levels of a certain chemokine receptor are altered.

[0198] In a particular embodiment, the fusion proteins provided by this invention can be of value in diagnostic applications. Diagnostic assays typically comprise detecting the formation of a complex resulting from the binding to said fusion protein to the corresponding chemokine receptor. For diagnosis purposes, the fusion protein provided by this invention may or may not be labeled. As mentioned previously, said fusion protein can be directly labeled with a suitable label. When they are not labeled, the fusion proteins provided by this invention can be detected using conventional methods, such as immunodetection or other binding assays *in vitro* known by persons skilled in the art. Said fusion proteins provided by this invention can also be used in research applications with non-diagnosis purposes.

Screening Methods

[0199] In another aspect, the fusion protein provided by this invention is used in a method for the identification of a compound with the ability to compete with said fusion protein for binding to a chemokine receptor *in vitro*, comprising:

a. providing at least one cell expressing the receptor corresponding to the fusion protein provided by this invention;

b. incubating said fusion protein in the absence or presence of different concentrations of the compound to be tested until equilibrium is reached; and

c. determining the amount of said protein bound to the corresponding receptor.

[0200] Said compound with the ability to compete with a fusion protein provided by this invention for binding to a chemokine receptor *in vitro* refers to a compound that imitates or inhibits the activity induced by a chemokine, or to a compound that binds to the chemokine receptor but does not imitate or inhibit the activity of said chemokine. The term "imitate", as used herein, means that said compound has an activity similar to that of a native chemokine; however, the effect induced by said analogous chemokine compound is not necessarily of the same magnitude as the activity induced by the native chemokine. Likewise, said analogous chemokine compounds can also comprise other parts in addition to those inhibiting or imitating the activity of a chemokine or binding to a chemokine receptor without causing or inhibiting the signaling thereof; as for example: peptides, antibodies or fragments thereof, fusion proteins, nucleic acid molecules, glucolipids, lipids, small chemical molecules, metals, salts, synthetic polymers (for example, polylactides and polyglycolide), surfactants and glycosaminoglycans, that are covalently or non-covalently bound to said analogous chemokine compound such that they do not alter the biological activity of said analog.

Methods of Treatment

[0201] In another aspect, the invention relates to a method for treating a disease characterized by the production of chemokines by the cells or tissues associated to said disease in a subject in need of treatment comprising administering a pharmaceutical composition comprising a fusion protein provided by this invention to said subject. The administration of the pharmaceutical composition of the invention can be carried out by conventional methods as has been described previously in relation to the administration of said pharmaceutical composition of the invention to a subject in need of treatment.

[0202] In a particular embodiment, the therapeutically effective amount of said fusion protein to be administered by the method according to what has been previously mentioned is comprised between 0.001 and 100 mg/kg of the body weight of the subject to be treated. The fusion protein of this invention will generally be administered one to three times a day (for example, one to three doses per day), each dose containing from approximately 0.001 mg/kg to approximately 100 mg/kg of body weight, although there will necessarily be variations depending on the weight and condition of the subject undergoing treatment, on the nature and severity of the disease for which the subject is being treated and the particular administration route chosen. However, a dose level comprised in the range of approximately 0.001 mg/kg to approximately 50 mg per kg of body weight per individual dose will be chosen as the most preferable dose. Nevertheless, there can be variations depending on the response of the individual subject to said medicament, as well as the type of pharmaceutical formulation chosen and the time period and interval in which the administration is carried out. The fusion protein dose can be repeated, depending on the condition of the patient and his/her evolution at time intervals (days, weeks, months) which will have to be established in each case by the specialist.

[0203] In another aspect, the invention relates to a method for the treatment and prevention of a pathology characterized by a growth or metastasis of tumor cells in a subject. Said method comprises administering to said subject in need of treatment a fusion protein provided by this invention or the pharmaceutical composition of the invention in a therapeutically effective amount for treating and/or preventing said pathology. In a particular embodiment, said metastasis pathology is selected from breast cancer, melanoma, ovarian cancer, pancreatic cancer, as well as metastasis in different organs, for example, liver, lung, bone or others.

[0204] In another particular embodiment, the administration of the fusion protein provided by this invention or pharmaceutical composition of the invention can be combined with other treatments according to the previously described medical conditions, for example, chemotherapy, radiotherapy, immunotherapy, surgery, etc., including alkylating agents, antimetabolites, sedatives, diuretic agents, antiviral agents, antibiotics, cytokines, nutritional supplements, etc. In a particular embodiment, the fusion protein will be administered in combination with a cytotoxic agent.

[0205] The administration of the cytotoxic agent or treatment as described previously as a combination therapy will be carried out simultaneously or sequentially in time to the administration of the protein or pharmaceutical composition of the invention.

[0206] In another particular embodiment, with the purpose of administering the fusion protein provided by this invention or the pharmaceutical composition of the invention according to the previously mentioned method, said administration will be carried out by at least one administration route selected from oral (e.g., oral, sublingual, etc.), parenteral (e.g., subcutaneous, intramuscular, intravenous, intra-articular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracerebellar, intracerebroventricular, intracolon, intracervical, intragastric,

intrahepatic, intramyocardial, intra-osseal, intrapelvic, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intra-rectal, intra-renal, intra-retinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesicular, etc.), vaginal, rectal, urethral, intranasal (pulmonary), topical, subcutaneous (e.g., transdermal, etc.), ophthalmic, auricular route etc., preferably by a parenteral route.

EXAMPLES

[0207] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0208] Accordingly, the invention is illustrated with the following non-limiting Examples.

EXAMPLE 1. Obtaining chemokine-Fc fusion proteins

1.1. Construction of chemokine-Fc recombinant molecules

1.1.1. Purification of peripheral blood lymphocytes

[0209] Peripheral blood lymphocytes (between 0.1 and 2 x 10^8 cells) of a donor were obtained by means of density gradient centrifugation Ficoll-Hypaque (Pharmacia-Pfizer, New York, NY, USA). The messenger RNA (mRNA) was extracted using the commercial product Tri-Reagent (Sigma, St. Louis, MO, USA) following the protocol recommended by the manufacturer. The integrity and purity of the extracted material was analyzed by means of

agarose/formaldehyde gels and analytically using the bioanalyzer Bio-Sizing (Agilent Technologies, Palo Alto, USA) for its quantification. After verifying the integrity of the sample, the resulting material was considered to be satisfactory in terms of quality and integrity and it was stored at -80 °C.

1.1.2. Obtaining human CCL2 and CCL5

[0210] Complementary DNA (cDNA) was synthesized from mRNA by means of the reverse transcriptase reaction, using deoxythymine octamers (oligo-dt) and the transcriptase *Superscript II RT* (Invitrogen, Carlsbad, CA, USA) as primers. The resulting material was used as a mold for amplifying by PCR the specific sequences encoding the chemokines CCL2 [MCP-1 (chemoattractant protein for monocytes-1); Genbank access no.: NM_002982] and CCL5 [RANTES (acronym of *Regulated upon Activation, Normal T cell Expressed and presumably Secreted)*; Genbank access no.: NM_002985]. The PCR amplification was carried out independently by means of conventional protocols, using several single chain oligonucleotides specific for each chemokine as primers. For the amplification of CCL2, the following primers were used: *MCP1-5Hind-* 5' CAT CAT AAG CTT GCC ACC ATG AAA GTC TCT GCC G 3' (SEQ ID NO: 45) and *MCP1-3Sal-* 5' CCA TTC GTC GAC AGT CTT CGG AGT TTG GG 3' (SEQ ID NO: 46); for the amplification of CCL5, the following primers were used: *RANTES-5Hind-* 5' CAT CAT AAG CTT GCC ACC ATG AAG GTC TCC GCG G 3' (SEQ ID NO: 47) and *RANTES- 3Sal-* 5' CCA TCT GTC GAC GCT CAT CTC CAA AGAG 3' (SEQ ID NO: 48).

[0211] In addition to containing specific sequences for the amplification of the chemokine genes, all the primers include specific restriction targets that are not present in the genes with the purpose of facilitating their cloning. The amplification cycles used for each pair of primers were: 94°C-30 s; 57°C-30 s and 72°C-45 s, after 40 cycles and in order to end the reaction, they were incubated at 72°C, 7 minutes.

1.1.3. Obtaining constructs of chemokine-Fc fusion proteins

[0212] In the obtaining of the gene constructs CCL2-Fc (SEQ ID NO: 23) and CCL5-Fc (SEQ ID NO: 35), the PCR products obtained in the previous step were used, these products were isolated in 2% agarose gels purified using the GeneClean II kit (Qbiogene, Montreal, Canada) following the manufacturer's instructions and their concentration was quantified by measuring the OD at 260/280 nm. The material obtained was digested with the restriction enzymes *Hind III* and *Sal I* (Roche, Basel, Switzerland), purified again and cloned in the pCDNA3 vector (Invitrogen, Carlsbad, CA, USA) in which the native Fc of a human IgG1 has previously been cloned. The clones obtained were analyzed by enzymatic digestion and the positive clones were verified by automatic DNA sequencing.

<u>1.1.4. Obtaining of chemokine-Fc variants by directed mutagenesis</u>

[0213] The generation of fusion protein variants of CCL2-Fc and CCL5 which has mutations, both precise and of deletion, in the chemokine domain and/or the Fc fragment, was carried out by directed mutagenesis using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The amplifications were carried out following the manufacturer's instructions, using the vector to be mutated as a mold. The amplified product obtained was incubated with the enzyme *DpnI* to digest the molecules that did not have the mutation/s. The digestion product was introduced by transformation by electroporation in XL1-Blue bacteria (Stratagene, La Jolla, CA, USA). After the transformation, the DNA of several clones was isolated and the presence of mutations was verified by automatic DNA sequencing.

Fusion proteins of general formula (Q_A-Y)$_2$

[0214] In the obtaining of the variants of fusion proteins CCL2-Fc and CCL5-Fc of general formula (Q_A-Y)$_2$ (Figure 2B) where Q_A is a variant of CCL2 or CCL5 and the polypeptide Y consists of the native Fc fragment of a human IgG1 immunoglobulin, using the constructs CCL2(1+9-76)-Fc (SEQ ID NO: 24), CCL2(P8A)-Fc (SEQ ID

NO: 25), CCL2(Y13A)-Fc (SEQ ID NO: 26) and CCL5(S24MP25A)-Fc (SEQ ID NO: 36), the following primers were used:

CCl2(1+9-76)-Fc:

M/DEL73-93- 5' CAA GGG CTC GCT CAG GTC ACC TGC TGT TAT AA 3' (SEQ ID NO: 49) and A-DEL73-93-5' TTA TAA CAG CAG GTG ACC TGA GCG AGC CCT TG 3'(SEQ ID NO: 50), designed to delete from the amino acid 2 to 8 of the native chemokine CCL2.

CCL2(P8A)-Fc:

MCP1-P8A- 5' AGA TGC AAT CAA TGC CGC AGT CAC CTG CTG TTA TA 3' (SEQ ID NO: 51) and MCP1-A-P8A- 5' TAT AAC AGCGGT GAC TGC GGC ATT GAT TGC ATC T 3' (SEQ ID NO: 52); designed so that the substitution of the proline 8 with an alanine can take place in the amino acid sequence of the native chemokine CCL2.

CCL2(Y13A)-Fc:

MCP-Y13A- 5' CCC CAG TCA CCT GCT GTG CAA ACT TCA CCA ATA GGA AGA TC 3' (SEQ ID NO: 53) and A-MCP-Y13A-5' GAT CTT CCT ATT GGT GAA GTT TGC ACA GCA GGT GAC TGG GG 3' (SEQ ID NO: 54); designed so that the substitution of tyrosine 13 with an alanine can take place in the amino acid sequence of the native chemokine CCL2.

CCL5(S24MP25A)-Fc:

S24MP25A- 5'CGC TCC TGC ATC TGC CAT GGC TAT TCC TCG GAC ACC 3' (SEQ ID NO: 55) y A-S24MP25A- 5' GGT GTC CGA GGA ATA TGC CAT GCA GAT GCA GGA GCG 3' (SEQ ID NO: 56), designed so that the substitution of serine 24 with a methionine and proline 25 with an alanine can take place in the amino acid sequence of the native CCL5 chemokine.

[0215] Likewise, for obtaining the variants of the fusion proteins CCL2-Fc and CCL5-Fc of general formula $(Q_A-Y)_2$ (Figure 2B) wherein Q_A is CCL2, CCL5 or a variant thereof and the polypeptide Y consists of a variant of the Fc fragment having a mutation FcR(-), using the constructs: CCL2-FcR(-)(SEQ ID NO: 27), CCL5-FcR(-)(SEQ ID NO: 37), CCL2(1+9-76)-FcR(-)(SEQ ID NO: 28), (CCL2(P8A)-FcR(-

)(SEQ ID NO: 29), CCL2(Y13A)-FcR(-)(SEQ ID NO: 30) and CCL5(S24MP25A)-FcR(-)(SEQ ID NO: 38), the following primers were used:

FCR-MUT- 5' GTG CCC AGC ACC TCC AGT AGC AGG GGG ACC GTC AGT 3' (SEQ ID NO: 57) and *A-FCR-MUT-* 5' ACT GAC GGT CCC CCT GCT ACT GGA GGT GCT GGG CAC 3'(SEQ ID NO: 58)

Said FcR(-) mutation consists of 3 mutations in the two Fc chains in those residues participating in the interaction with the receptor FcγR: E233P/L234V/L235A (Isaacs JD et al. 1998).

Fusion proteins of general formula Q_A-Y

[0216] In the obtaining of the variants of fusion proteins CCL2-Fc and CCL5-Fc of general formula Q_A-Y (Figure 2C) where Q_A is CCL2, CCL5 or a variant thereof and the polypeptide Y consists of a variant of the Fc fragment in which the cysteines at positions 226 and 229 have been substituted with serines or with alanines (C226S/C229S or C226A/C229A), the following constructs were used: CCL2-HFc(Ser) (SEQ ID NO: 32), CCL2(1+9-76)-HFc(Ser) (SEQ ID NO: 34), CCL2-HFc(Ala) (SEQ ID NO: 31) and CCL2(1+9-76)-HFc(Ala) (SEQ ID NO: 33). The following primers were used for this:

CCL2-HFc(SER), CCL2(1+9-76)-HFc(SER):

MONOFCSER- 5' CAA AAC TCA CAC AAG CCC ACC GAG CCC AGC ACC TGA AC 3' (SEQ ID NO: 59) and

A-MONFCSER- 5'GTT CAG GTG CTG GGC TCG GTG GGC TTG TGT GAG TTT TG 3' (SEQ ID NO: 60)

CCL2-HFc(ALA), CCL2(1+9-76)-HFc(ALA):

MONOFCALA- 5' CAA AAC TCA CAC AGC CCC ACC GGC CCC AGC ACC TGA AC 3' (SEQ ID NO: 61) and

A-MONFCALA- 5' GTT CAG GTG CTG GGG CCG GTG GGG CTG TGT GAG TTT TG 3'(SEQ ID NO: 62)

[0217] Said mutation in the cysteines 226 and 229 allows avoiding the formation of disulphide bridges between chains and thus prevents dimerization of Fc.

Heterodimeric fusion proteins of general formula (Q_A-Y)-(Q_B-Y) and

Q_A- (Y)$_2$

[0218] In the obtaining of heterodimeric variants of fusion proteins CCL2-Fc and CCL5-Fc of general formula (Q_A-Y)-(Q_B-Y) (Figure 2A) where Q_A is CCL2 or a variant thereof and Q_B is CCL5 or a variant thereof, for example, in the fusion protein denominated (CCL2-HFc)-(CCL5-HFc), *"knob" or "hole"* mutations were performed respectively, in each of the Fc (HFc) chains in the residues described by Merchant M et al, 1998. Said "knob" and "hole" mutations can be, without distinction, in the HFc chain bound to CCL2 or in the HFc chain bound to CCL5, therefore said fusion protein can be obtained from a construct comprising the sequence encoding the CCL2-Fc KIH- *knob* chain (SEQ ID NO: 39) and from another construct comprising the sequence encoding the CCL5-Fc KIH- *hole* chain (SEQ ID NO: 42) or vice versa, from a construct comprising the sequence encoding the CCL2-Fc KIH- *hole* chain (SEQ ID NO: 41) and from a construct comprising the sequence encoding the CCL5-Fc KIH-*knob* chain (SEQ ID NO: 40). Said *"knob into hole"* modification favors heterodimerization between chains over the homodimerization thereof. The following primers were used to introduce said modification:

HFc *KNOB* CHAIN:

C1-Y349C- 5' GAA CCA CAG GTG TGC ACC CTG CCC C 3' (SEQ ID NO: 63) and A-C1-Y349C- 5' GGG GCA GGG TGC ACA CCT GTG GTT C 3' (SEQ ID NO: 64);

C1-T366W- 5' GAA CCA CAG CCT GTG GTG CCT GGT CAA AGG CT 3' (SEQ ID NO: 65) and A-C1-T366W- 5' AGC CTT TGA CCA GGC ACC ACA GGC TGA CCT GGT TC 3' (SEQ ID NO: 66).

HFc *HOLE* CHAIN:

C2-D356C- 5' GCC CCC ATC CCG GTG TGA GCT GAC CAA GAA C 3' (SEQ ID NO: 67) and A-C2-D356C- 5' GTT CTT GGT CAG CTC ACA CCG GGA TGG GGG C 3' (SEQ ID NO: 68);

T366SL368A- 5' GGT CAG CCT GTC CTG CGC AGT CAA AGG CTT CTA TCC C 3' (SEQ ID NO: 69) and

A-C2-TS/LA- 5' GGG ATA GAA GCC TTT GAC TGC GCA GGA CAG GCT GAC C 3' (SEQ ID NO: 70);

C2-Y407V- 5'CGG CTC CTT CTT CCT CGT AAG CAA GCT CAC CGT GG 3' (SEQ ID NO: 71) A-C2-Y407V- 5' CCA CGG TGA GCT TGC TTA CGA GGA AGA AGG AGC CG 3'(SEQ ID NO: 72).

[0219] In the obtaining of $(CCL2)_1$-Fc, in addition to the aforementioned primers for obtaining a variant of the Fc fragment, the following primers were used with the aim of maintaining the signal peptide of the CCL2 chemokine in one of the chains and eliminating the rest of the secreted protein CCL2:

$(CCL2)_1$-HFc:

MCP1-5Hind- 5' CAT CAT AAG CTT GCC ACC ATG AAA GTC TCT GCC G 3' (SEQ ID NO: 45)

MCPps/FC3'-5' GTG AGT TTT GTC GAC AGC GAG CCC TTG G 3'(SEQ ID NO: 73);

MCPps/FC5'- 5' CCA AGG GCT CGC TGT CGA CAA AAC TCA C 3' (SEQ ID NO: 74) and

FC/VECTOR3'- 5' ATG CTC GAG CGG CCG CCG CAC TCA TTT ACC 3' (SEQ ID NO: 75)

[0220] Said fusion protein $(CCL2)_1$-Fc can be obtained from a construct comprising the sequence encoding the CCL2ps-Fc-KIH- *knob* chain (SEQ ID NO: 43) and from another construct comprising the sequence encoding the CCL2-Fc-KIH- *hole* chain (SEQ ID NO: 41) or vice versa, from a construct comprising the sequence encoding the CCL2ps-Fc-KIH- *hole* chain (SEQ ID NO: 44) and from another construct comprising the sequence encoding the CCL2-Fc-KIH- *knob* chain (SEQ ID NO: 39).

<u>1.2. Production of recombinant proteins CCL2-Fc and CCL5-Fc</u>

[0221] The day before transfection, human embryonic kidney cells HEK-293 (ATCC TIB202, Rockville, MD, USA) were seeded at a concentration of approximately 20000 cells/cm^2 and were transfected with JetPei (Qbiogene, Montreal, Canada) following the manufacturer's instructions. For every 10^6 cells, 2 μg of plasmid

DNA from different constructs was used. After 5 hours, the transfection medium was changed for serum-free culture medium HYQ-SFM4HEK293 (Hyclone, Logan, UT, USA).

[0222] In prior transfections, the expression of different proteins was analyzed after 24, 48 and 72 hours (h) (more specifically, the proteins CCL2-Fc and CCL5-Fc as described in Figure 3A and 3B), observing a maximum expression after 72 h, therefore it was determined that the collection of supernatant should be carried out 72 h after the cell transfection.

<u>1.2.1. Purification of the chemokine-Fc fusion proteins</u>

[0223] The purification of the fusion proteins was carried out by a Hi-Trap protein G column (Amersham-GE, USA). Before passing through the column, the supernatant collected from the transfections was filtered through a 0.45 micron filter with low binding to protein (Millex, Millipore, Billerica, MA, USA) and it was applied to the column previously equilibrated with phosphate-buffered saline (PBS), at a speed of 3 ml/min. After carrying out the load, the column was washed with PBS to remove the non-specifically bound proteins. In order to elute the protein adhered to the column, 100 mM glycine-HCl buffer pH 2.9 was used and the eluted fractions were immediately neutralized with Tris-HCl pH 8.8.

[0224] The protein obtained was concentrated in Amicon Y-10 columns (Millipore, Billerica, MA, USA) and subsequently quantified by means of ELISA. The apparent molecular weight was determined using a mixture of pre-stained proteins with a known molecular weight as a standard (New England Biolabs, Beverly, MA, USA) by means of the Western Blot technique.

<u>1.2.2. Verification of the molecular weight of the purified proteins</u>

[0225] Both the transfection supernatants and the purified fusion proteins were subjected to migration in SDS/PAGE gels at 12,5% bis/acrylamide (BioRad, Hercules, CA, USA) to verify that

the apparent molecular weight of the proteins corresponded to the dimer (Figures 4A, 4B, 4C, 4D, 4E and 4F). Once migrated, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA) previously activated with methanol. The PVDF membranes were blocked with 5% skimmed milk powder in PBS-0.1% Tween-20 and later incubated with the suitable antibody for 1 h at 37°C. After several washings with PBS-0.1 % Tween-20, a second incubation was carried out with corresponding mouse anti-IgG antibodies or streptavidin-HRP labeled with peroxidase. After washing them, the membranes were developed with the ECL chemoluminiscence kit (Amersham-GE, USA).

[0226] Table 1 shows the generated chemokine-Fc fusion proteins, classified according to their general structure.

SEQ ID NO: 1-22 show the amino acid sequence of the Fc-fusion proteins of the invention. In figure 12, the structure of these constructs is clearly indicated. For example, in CCL2-Fc (SEQ ID NO: 1), the first 23 amino acids correspond to the signal peptide of CCL2 that allows the fusion protein to be secreted, amino acids 24-99 correspond to the CCL2 chemokine and finally amino acids 100-327 correspond to the Fc fragment of an IgG1 antibody.

[0227] **Table 1**

Q_A-Y	SEQ ID NO:
CCL2-HFc(Ser)	10
CCL2-HFc(Ala)	9
CCL2(1 +9-76)-HFc(Ser)	12
CCL2(1 +9-76)-HFc(Ala)	11
(Q_A-Y)$_2$	**SEQ ID NO:**

CCL2-Fc	1
CCL2(1+9-76)-Fc	2
CCL2(P8A)-Fc	3
CCL2(Y13A)-Fc	4
CCL5-Fc	13
CCL5(S24MP25A)-Fc	14
CCL2-FcR(-)	5
CCL2(1 +9-76)-FcR(-)	6
CCL2(P8A)-FcR(-)	7
CCL2(Y13A)-FcR(-)	8
CCL5-FcR(-)	15
CCL5(S24MP25A)-FcR(-)	16
CCL3-FcR(-)	80
CCL4-FcR(-)	81
CXCL8-FcR(-)	82
CXCL12-FcR(-)	83
(Q_A-Y)-(Q_B-Y)	**SEQ ID NO:**
(CCL2-HFc)-(CCL5-HFc)	*
Q_A-(Y)$_2$	**SEQ ID NO:**
(CCL2)$_1$-Fc	**

* (CCL2-HFc)-(CCL5-HFc) is a heterodimer formed by the CCL2-Fc KIH- *knob* chain (SEQ ID NO: 17) and by the CCL5-Fc KIH- *hole* chain (SEQ ID NO: 20) or, alternatively, by the CCL2-Fc KIH- *hole* chain (SEQ ID NO: 18) and the CCL5-Fc KIH- *knob* chain (SEQ ID NO: 19).

** $(CCL2)_1$HFc is a fusion protein formed by the CCL2ps-Fc-KIH-*knob* chain (SEQ ID NO: 21) and by the CCL2-HFc *hole* chain (SEQ ID NO: 18) or, alternatively, by the CCL2ps-Fc-KIH- *hole* chain (SEQ ID NO: 22) and by the CCL2-HFc *knob* chain (SEQ ID NO: 17)

1.2.3. Quantification of the concentration of purified proteins

[0228] The purified protein obtained was quantified by a specific enzymatic immunodetection assay (ELISA). For the immunoassay in plates, a specific anti-human Fc antibody (Jackson ImmunoResearch, Soham, UK) was used as a supporting surface at a concentration of 2,5 µg/ml. The secondary antibody used was a specific antihuman Fc-HRP (Jackson ImmunoResearch, Soham, UK) used according to the manufacturer's instructions.

[0229] Several dilutions of the transfection supernatant, of the purified and concentrated proteins as well as of the standard curves carried out with the complete IgG molecule (Pierce, Rockford, IL, USA) and purified human Fc fragment (Chemicon, Temecula, CA, USA) were incubated to quantify the presence of the fusion protein Fc. The concentration of the stock of purified proteins was obtained when the OD 498 nm data of the ELISA was extrapolated against that obtained in the standard curves.

EXAMPLE 2. Determination of the specificity of the CCL2-Fc fusion proteins by the CCR2 receptor

2.1. Cell migration assays

[0230] In the chemotaxis assays, 24 well migration chambers (Costar, USA) with 5 micron pore size inserts were used, which are ideal for assays carried out with THP1 cells (ATCC TIB202 Rockville, MD, United States) and/or MonoMac-1 (DSM ACC252, Braunschweig, Germany).

[0231] The migration assays carried out show that the protein CCL2-Fc is CCR2 receptor-specific. The monocytic cell line THP1 expressing said receptor was used for these assays. The cells were cultured at the suitable concentration so that they were in exponential phase at the time of the assay. The cells were

counted, washed twice and placed in the inserts of the migration chamber (2.5 x 10^5 cells in 100 µl). The protein CCL2-Fc was placed in the lower part of the inserts diluted in 0.6 mL of GEYS (Sigma, St. Louis, MO, USA) + 0.5% BSA and at the same time, wells with CCL2-Fc (5 to 20 nM) and anti-MCP1 antibody (Peprotech, Rocky Hill, NJ, USA) were prepared at 0.5 µg/well. In parallel within the same assay, the cells which were going to migrate were incubated with 0.5 µg of anti-CCR2 antibody (1D9) for 15 minutes at 37°C, later adding the cells to the corresponding migration inserts. All the points were carried out in duplicate. The cells were incubated for 180 to 240 minutes at 37°C with 5% CO_2, after which the inserts were removed and the cells which had migrated to the lower part of the chamber were counted by means of flow cytometry. The migration index was calculated as the relation between the migrated cells in response to different stimuli and the cells migrated in the absence of stimulus.

[0232] Figure 5 shows a migration experiment carried out with THP1 cells in which it is observed that the cells migrate specifically through the CCR2 receptor. In this experiment, it is observed that THP1 cells migrate against different concentrations of CCL2-Fc (5, 10 and 20 nM) and this migration decrease if the cells which are going to migrate are incubated with 0.5 µg of anti-CCR2 antibody (1D9), indicating that the receptor blocking prevents the CCL2 chemokine part of the fusion protein CCL2-Fc from binding to the CCR2 receptor of which it is the specific ligand. On the other hand, the incubation of the protein CCL2-Fc with an antibody blocking its chemokine domain decreases cell migration, indicating that the fusion protein domain is the one that provides the molecule with its specificity against the CCR2 receptor.

2.2. Calcium mobilization assays

[0233] Calcium mobilization assays were carried out to show the specificity of the fusion protein CCL2-Fc to the CCR2 receptor.

[0234] The changes in calcium mobilization were determined by using the fluorescent probe Fluo-3 AM (Calbiochem, San Diego, CA, USA). The THP1 cells (2.5 x 10^6 cells/ml) were resuspended in RPMI culture medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Cambrex, Rockland, ME, USA) and incubated with 10 µl of Fluo-3 AM (300 µM in DMSO) for 20 minutes at 37°C with continuous stirring. The cells were washed twice in RPMI medium with 10 mM of HEPES and were resuspended at a concentration of 0.5 x 10^6 cells/ml in RPMI medium with HEPES, 0,5% BSA (bovine serum albumin) and 2 mM Cl_2Ca.

[0235] Until the assay was carried out, the cells were aliquoted, maintained at 4°C and prior to ligand addition they were taken to a temperature of 37°C. The response of calcium release was determined by flow cytometry, measuring fluorescence at 525 nm.

[0236] As shown in figure 6A, the cells release calcium in response to the fusion protein CCL2-Fc (5 nM). As observed in Figure 6B, there is no intracellular calcium release when purified human Fc fragment (2 nM) is added; this shows that the Fc part of the fusion protein is not responsible for calcium mobilization. On the other hand, Figure 6C shows another assay in which the CCR2 receptor of THP1 cells loaded with 5 µg of anti-CCR2 antibody (1D9) was blocked and the subsequent addition of the protein CCL2-Fc (20 nM) did not produce intracellular calcium release, indicating that the binding of the fusion protein CCL2-Fc is specifically carried out by the CCR2 receptor.

EXAMPLE 3. CCL2-Fc and its variant CCL2 (1+9-76)-Fc inhibit the action of the chemokine CCL2

3.1. Migration inhibition assays

[0237] The chemotaxis experiments were carried out as previously indicated in Example 2. Migration assays were carried out with THP1 cells using CCL2 as a migrating stimulus at

concentrations of 2.5 and 5 nM, which are the concentrations at which CCL2 has the greatest chemoattractant activity for these cells. The inhibitory effect of the fusion proteins CCL2-Fc and its variant CCL2 (1+9-76)-Fc on the migration induced by CCL2 was determined by adding different concentrations (0.1-12.5 nM) of the proteins to the THP1 cells that were going to migrate, the mixture being incubated in Eppendorf tubes for 10 minutes at 37°C, after which the cells were added to the migration inserts.

[0238] The migration of the cells with the stimulus produced by chemokine CCL2 both at 2.5 and at 5 nM (positive control) concentration can be observed in Figure 7. When the THP1 cells are incubated with the fusion proteins CCL2-Fc and its variant CCL2 (1+9-76)-Fc at different concentrations, the CCR2 receptor is blocked and the migration against the CCL2 stimulus decreases, indicating the inhibitory activity of these proteins against the specific stimulus of CCL2.

<u>3.2. Desensitization produced by the fusion proteins in calcium mobilization assays</u>

[0239] Calcium mobilization assays were carried out as previously described in Example 2. As shown in Figure 8, intracellular calcium release curves were made at different concentrations (1-20 nM) of both recombinant CCL2 (positive control) and fusion proteins CCL2-Fc and its variant CCL2(1+9-76)-Fc, observing that these proteins are capable of mobilizing intracellular calcium at a minimum concentration of 3 nM. CCL2 was added in these same assays observing that calcium was not released from concentrations exceeding 3 nM, therefore, the mobilization that should have occurred as a result of the addition of CCL2 was inhibited by these proteins. These data indicate that the desensitization of CCR2 against the stimulus that should have been caused by its natural ligand CCL2 was produced by the specific blocking of the receptor by the previously added fusion proteins. In some assays, CXCL12 (SDF-1), the specific receptor of which is

CXCR4, has been added to show that the stimulus caused by the proteins CCL2-Fc and CCL2(1+9-76)-Fc is CCR2-specific and other receptors are not affected in the response to its specific ligand. Likewise, in one of the assays, ionomycin has been added at 10 nM to verify whether the calcium load of the cells used in the assay was optimum.

EXAMPLE 4. Determination of the concentration in which the fusion proteins exert 50% of their inhibitory activity (IC50)

[0240] The migration inhibition assays were carried out as described previously in Example 3, although in this case, the cell line used was the monocyte line MonoMac-1.

[0241] Previously, the chemotactic response curve of the MonoMac cells was determined at different concentrations of CXCL12. Later, chemotaxis assays were carried out to determine the IC50 of the fusion proteins using the concentration of CXCL12 corresponding to 50% with respect to the maximum migration point induced by said chemokine as the migrating stimulus for the MonoMac-1 cells. The cells that were going to be used in the migration were incubated at different concentrations (0.06-10 nM) of the fusion proteins CXCL12-FcR(-), after which the cells were added to the migration inserts and the assay was begun. In Figure 9 it can be observed that the incubation with fusion proteins produced the inhibition of the migration of MonoMac cells to the stimulus produced by the chemokine CXCL12. The concentration for which the protein CXCL12-FcR(-) showed 50% of its inhibitory effect is approximately 10 nM.

[0242] The same migration inhibition assays were also carried out with the fusion protein CCL3-FcR(-)and the concentration for which protein showed 50% of its inhibitory effect is more than 10 nM.

EXAMPLE 5. Model of bleomycin-induced lung injury in mice

[0243] An experiment will be performed in bleomycin-induced lung injury in a mouse model to determine the effect of the molecules described herein.

[0244] Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal lung disease characterized by the proliferation of fibroblast and deposition of extracellular matrix, including fibrillar collagens, fibronectin, elastic fibers, and proteoglycans. Experimental procedure implies the development of an experimental pulmonary fibrosis that include two phases; a persistent inflammatory phase and a sequential fibrotic phase.

[0245] Bleomycin is suspended in PBS and is administered endotracheally via instillation at a dose of 0.075U/ml. Seven days after Bleomycin treatment mice are randomly distributed in two groups: one group receives a daily intraperitoneally injection of vehicle, and the other group receives a daily intraperitoneally jof the fusion protein study at the adequate doses. The treatments are extended for 15 days.

[0246] At the end of the study, 28 days after the injury, mice are sacrificed and the therapeutic effect of the molecules described herein are tested by: 1) Counting the cell number and cell classification in bronchoalveolar lavage, 2) semi-quantitative histological analysis in lung tissue by Ascroft score, 3) expression of cytokines and chemokines will be evaluated in lung tissue, and 4) total lung collagen be determine by as hydroxyproline content.

These findings may suggest that the fusion proteins of the present invention will improve bleomycin-induced pulmonary fibrosis in mice by inhibiting cell infiltration and matrix deposition.

EXAMPLE 6. Model of arthritis induced by collagen type II in mice

[0247] An experiment will be performed in type II collagen induced arthritis in a mouse model to determine the prophylactic effect of the molecules described in the present invention.

[0248] Rheumatoid arthritis is a destructive inflammatory disease characterized by the recruitment and sequestration of various inflammatory cells types to the joints. During the development of the disease, the two to three cell thick layers lining the synovium proliferates, and the synovial membrane becomes inflamed under the influence of the newly arrived leukocytes. Chemokines are a superfamily of small pro-inflammatory proteins that selectively activate and recruit leukocytes to the site of inflammation.

[0249] A widely used experimental model of the pathogenic mechanism of human rheumatoid arthritis is the type II collagen induced arthritis model in susceptible mice. This model shares many of the cellular and humoral events found in human disease including synovial inflammation.

[0250] In order to elucidate the effect of the fusion proteins of the invention in this model, arthritis will be induced in male DBA/1 mice by immunization with native type II bovine collagen emulsified with an equal volume of complete Freund's adjuvant (CFA). 200 ug of CII is injected intradermally at the base of the tail of the mice. Twenty-one days after immunization, the animals are challenged with 200ug of CII intraperitoneally in PBS. Animals are divided in two groups: one group receives a daily intraperitoneally injection of vehicle. The other group receives a daily intraperitoneally injection of the fusion protein at the adequate doses. Treatment begins at the time of CII challenge and is continued for 10 consecutive days. Mice are evaluated every day for signs of arthritis based on adequate scores. At the end of the study, 40 days after arthritis induction, the mice are sacrificed and the therapeutic effect of the molecules described herein are tested as: 1) semiquantitative histological analysis in paw tissue using appropriate score, 2) expression of cytokines and chemokines will be evaluated in joint tissue, and 3) inmunohistology will be done to determine and localize some

inflammatory molecules (markers of joint inflammation and destruction).

[0251] These findings may suggest that the prophylactic administration of the molecules described herein could improve the inflammatory process in an arthritis model. Our study may suggest that the effective targeting of chemokines -triggered events with the fusion molecules described herein can be of therapeutic importance in the treatment of rheumatoid arthritis.

EXAMPLE 7. Model of arthritis induced by collagen type II in mice

[0252] We will utilize the results obtained from Example 2 and the induction of arthritis will be done as described above. When animals develop overt arthritis with a minimal clinical score of 2, they will randomly be assigned to treatment groups and given either vehicle or the adequate doses of fusion protein for 14 consecutive days. On the day after the initial challenge, the paws of the animals are prepared for histological and inmunohistologic evaluation, as in Example 2. Our studies may suggest that the effective targeting of chemokines -triggered events with the fusion molecules described herein may have therapeutic importance in the treatment of rheumatoid arthritis.

EXAMPLE 8. In vivo assessment of a fusion protein of the invention in Inflammatory Bowel Disease

Materials and Methods

Ulcerative Colitis Model

[0253] Ulcerative colitis is induced in Sprague Dawley rats (7-8 weeks old) by the administration of a solution in which 90 mg of trinitrobenzenesulfonic acid (TNB) is dissolved in 1.5 ml. of 20% ethanol. Certain groups of rats are treated with various doses of a chemokine fusion protein of the invention and other groups are treated with a vehicle control. In these studies, the preferred route of administration of a **fusion protein of the invention** is

by catheter to deliver the compound directly to the colon. Most preferably, a rubber catheter such as a Nelaton catheter No. 8 is used (Rush Company, West Germany). The compound is preferably introduced about 6 cm from the rectum in the rat. One of skill in the art will be familiar with the use of such catheters to deliver compounds to the desired site in rats of varying ages and weights and in other experimental animals. During the experiments rats are clinically evaluated daily, and presence or absence of diarrhea is monitored.

[0254] At one to two weeks after induction of colitis, the rats are sacrificed by decapitation and evaluated for severity of colonic lesions and general colonic pathology to evaluate the development of ulcerative colitis. The colon is rapidly removed, opened, rinsed in saline, blotted gently, weighed and fixed in 10% formalin. Standardized sections of ileum, jejunum, duodenum, stomach, liver, pancreas, kidneys and lungs are also fixed, and processed for histologic examination. Additional sections from grossly involved and uninvolved areas of colon, ileum and jejunum are frozen and subsequently homogenized for the determination of colonic myeloperoxidase activity by the method of Bradley et al. (Bradley, P. P., et al., J. Invest. Dermatol. 78:206-209 (1982)) using 0.0005% hydrogen peroxide as a substrate. This enzyme, located mainly in the azurophilic granules of polymorphonuclear leukocytes is used as a quantitative index of inflammation (Morris, G. P., et al., Gastroenterology 96:795-803 (1989); Bradley, P. P., et al., J. Invest. Dermatol. 78:206-209 (1982); Krawisz, J. E., et al., Gastroenterology 47:1344-1350 (1985)).

[0255] For morphologic studies at the light microscopy level 2-4 mm long tissue sections of tissue are fixed in 10% buffered (pH7) formalin, dehydrated and embedded in paraffin or in the J8-4 plastic embedding medium. Sections (1-5 um) from all organs are stained with hematoxylin and eosin (H&E) and, in addition,

sections from stomach and duodenum are also stained with the periodic acid-Schiff (PAS) technique.

Morphometric analysis of colonic lesions is performed by stereomicroscopic planimetry (Szabo, S., et al., J. Pharm. Methods 13:59-66 (1985); Szabo, S., et al., Gastroenterology 88:228-236 (1985); Szabo, S., et al., Scand. J. Gastroenterol. 21 Suppl.:92-96 (1986)). In addition, "damage scores" 0-5 are calculated using a combination of gross and histologic assessment of the extent of TNB-induced colonic lesions (Morris, G. P., et al., Gastroenterology 96:795-803 (1989)). Thus, there are four quantitative endpoints in evaluating the experimental colonic lesions: planimetry (mm^2) of involved colon, damaged score (grades 0-5) derived from gross and histologic evaluation, colon weight (Calkins, B. M., et al., Epidemiol. Rev. 8:60-85 (1986)) indicating edema, inflammatory infiltrate and tissue proliferation, as well as myeloperoxidase activity quantitatively reflecting the intensity of inflammation.

[0256] Tissue samples from colon and ileum from rats killed up to 2 days after IA or NEM are fixed in Karnovsky's fixative for electron microscopy, dehydrated in graded ethanol, embedded, cut and stained for examination by transmission electron microscopy as described (Trier, J. S., et al., Gastroenterology 92:13-22 (1987)).

[0257] For biochemical studies, the tissue (total thickness, mucosa and muscle separated in certain experiments) is either homogenized with a Tekmar homogenizer, or kept frozen for up to two weeks.

[0258] For statistical evaluation, the results are stored and analyzed by computer. The statistical significance of differences of the group values are calculated (for parametric data) by two-tailed Student's t-test or (with parametric statistics) by the

Mann-Whitney test or the Fisher-Yates Exact Probability Test.

EXAMPLE 9. In Vivo Assessment Of A Fusion Protein Of The Invention In A Multiple Sclerosis Model

[0259] Lysolecithin Induced Demyelination

For these experiments, 12 week old SJL/J mice are anesthetized with sodium pentobarbitol and a dorsal laminectomy is performed in the upper thoracic region of the spinal cord. A 34 guage needle attached to a Hamilton syringe is used to inject 1μl of a 1% solution of lysolecithin directly into the dorsolateral aspect of the cord. Animals are killed on day 21 post injection and the injected region of the spinal cord is removed and processed for morphological evaluation.

[0260] As a second model of demyelination, intraspinal injection of lysolecithin is used. Twelve-week-old SJL/J mice are anesthetized by intraperitoneal injection of sodium pentobarbitol (0.08 mg/g). Dorsal laminectomies are performed on the upper thoracic region of the spinal cord and lysolecithin (L-lysophosphatidylcholine) (Sigma, St. Louis, MO) is injected as described (Pavelko, K.D., van Engelen, B.G. & Rodriguez, M. (1998) J. Neurosci. 18, 2498_2505). Briefly, a 34 gauge needle attached to a Hamilton syringe mounted on a stereotactic micromanipulator is used to inject 1% solution of lysolecithin in sterile PBS (pH 7.4) with Evan's blue added as a marker. The needle is inserted into the dorsolateral part of the spinal cord, 1 μl of lysolecithin solution is injected, and then the needle is slowly withdrawn. The wound is sutured in two layers, and mice are allowed to recover. The day of lysolecithin injection is designated day 0.

[0261] Seven days after lysolecithin injection, mice are treated with a fusion protein of the invention as a bolus intraperitoneal injection or intravenously. Initially a dose response study will

be done to establish the most effective dose for use in this animal model. Control mice are treated with bolus intraperitoneal or intravenous injection of vehicle control. Three weeks and five weeks after the lysolecithin injection, mice are sacrificed and one mm thick sections are prepared. The araldite block showing the largest lysolecithin induced demyelination lesion is used for quantitative analysis. The total area of the lesion is quantitated using a Zeiss interactive digital analysis system. The total number of remyelinated fibers are quantitated using a Nikon microscope/computer analysis system. The data is expressed as the number of remyelinated axons/mm^2 of lesion.

[0262] Lysolecithin treated mice are given various doses of a a fusion protein of the invention on days 0, 3, 7, 10, 14, and 17 after lysolecithin injection. Animals are killed on day 21 after lysolecithin injection. PBS or vehicle controls serve as negative controls.

EAE Model

[0263] Experimental allergic encephalomyelitis (EAE) is a T cell mediated autoimmune disease of the central nervous system (CNS). Disease can be induced in susceptible strains of mice by immunization with CNS myelin antigens or alternatively, disease can be passively transferred to susceptible mice using antigen stimulated CD4+ T cells [Pettinelli, J. Immunol. 127, 1981, p. 1420]. EAE is widely recognized as an acceptable animal model for multiple sclerosis in primates [Alvord et al. (eds.) 1984. Experimental allergic encephalomyelitis--A useful model for multiple sclerosis. Alan R. Liss, New York]. The effects of administration of a fusion protein of the invention on induction of EAE following the adoptive transfer of lymphocytes from immunized mice restimulated *in vitro* with a synthetic peptide of myelin proteolipid protein (PLP) is studied.

[0264] Adoptive Transfer of LNC sensitized by in PLP in vitro and in vivo. Female SJL/J mice (7-10 wks) are purchased from The Jackson Laboratory, and are housed 5 to a cage and are fed standard rodent chow diet with water ad libitum. Mice are divided into groups and certain groups are treated with vehicle control (PBS), other groups are treated with various doses of a fusion protein of the invention. Mice are then immunized in two sites on the flank with 150 μg of mouse PLP peptide comprising residues 139-151. PLP was administered in 200 μl of Complete Freunds adjuvant containing 2 mg/ml Mycobacteria Tuberculosis H37RA (Difco). On the day of immunization mice are injected intravenously with 0.75 x 10^{10} Bordatella pertussis bacilli (Massachusetts Public Health Laboratories, Boston, Mass.). Ten days after immunization, spleens and lymph nodes (popliteal, axillary and brachial) are harvested and the cells are resuspended in RPMI-1640 containing 10% FBS (Hyclone), 5 x 10^{-5} M 2-Mercaptoethanol, 100 μg/ml streptomycin and 100 U/ml penicillin. PLP is added to the cultures at 2 μg/ml. After 96 hours, the cells are harvested, washed twice and are injected i.p. into naive SJL/J mice. For clinical Evaluation of Disease; mice are observed for clinical signs of EAE and are scored on a scale of 0 to 3 as follows:

0.5--Distal limp tail
1.0--Complete limp tail
1.5--Limp tail and hind limb weakness (unsteady gait)
2.0--Partial hind limb paralysis
3.0--Complete bilateral hind limb paralysis

[0265] Literature

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What is claimed is:

1. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A-Y)-(Q_B-Y)$;

(b) Q_A-Y; or

(c) $Q_A-(Y)_2$

wherein

Q_A is CCL2 or a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

2. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A-Y)-(Q_B-Y)$;

(b) Q_A-Y; or

(c) $Q_A-(Y)_2$

wherein

Q_A is CCL5 or a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

3. A fusion protein comprising (i) a chemokine or a variant

thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein

Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A);

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

4. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein

Q_A is a variant of the CCL5 chemokine, designated CCL5(S24MP25A);

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

5. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)-(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein

Q_A is a CC chemokine selected from the group consisting of: CCL2, CCL5, CCL3 and CCL4 or variants thereof;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

6. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)-(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A\text{-}(Y)_2$

wherein

Q_A is a variant of a CC chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A), CCL2(Y13A) and CCL5(S24MP25A);

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

7. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)-(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$; or

(c) $Q_A-(Y)_2$

wherein

Q_A is a CXC chemokine selected from the group consisting of: CXCL12 and CXCL8 or variants thereof;

Q_B is a chemokine or a chemokine variant different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

8. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A-Y)-(Q_B-Y)$;

wherein

Q_A is CCL2 or a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is CCL5 or a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin.

9. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A-Y)-(Q_B-Y)$;

wherein

Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A);

Q_B is a variant of the CCL5 chemokine, designated CCL5(S24MP25A); and

Y is a polypeptide comprising an optionally modified portion

of the constant region of an immunoglobulin.

10. A fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula $(Q_A\text{-}Y)_2$, with the condition that:

d) when Q_A is CCL1 from mouse, then Y is not the fragment Fc of the human IgG_1 immunoglobulin; and

e) when Q_A is CCL19 from mouse, then Y is not the fragment Fc of the mouse IgG_{2b} immunoglobulin;

wherein

Q_A and Y have the meanings indicated previously.

11. A fusion protein according to claim 10 of general formula $(Q_A\text{-}Y)_2$, in which Q_A is a CC chemokine or a variant thereof or a CXC chemokine or a variant thereof; and the polypeptide Y comprises a native Fc fragment.

12. A fusion protein according to claim 11, in which Q_A is a CC chemokine selected from the group consisting of CCL2, CCL5, CCL3 and CCL4, or a variant thereof.

13. A fusion protein according to claim 11 in which Q_A is a variant of CCL2 selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or a variant of CCL5, designated CCL5(S24MP25A).

14. A fusion protein according to claim 12 in which Q_A is a CXC chemokine selected from the group consisting of CXCL12 and CXCL8, or a variant thereof.

15. The fusion protein according to any one of claims 1-10, wherein said polypeptide Y comprises a portion of the constant

region of an immunoglobulin molecule.

16. The fusion protein according to claim 15 wherein said immunoglobulin molecule is of human origin.

17. A fusion protein according to claim 15, wherein said polypeptide Y comprises at least one domain of the constant region of the heavy chain of an immunoglobulin molecule.

18. A fusion protein according to claim 17, in which said polypeptide Y comprises at least one domain of the heavy chain of an immunoglobulin selected from the CH2 domain and the CH3 domain of the heavy chain of an immunoglobulin.

19. A fusion protein according to claim 18, in which said polypeptide Y comprises the CH2 and CH3 domains of the heavy chain of an immunoglobulin.

20. A fusion protein according to claim 18, in which said polypeptide Y further comprises the hinge region of an immunoglobulin or a fragment thereof.

21. A fusion protein according to any one of claims 1-10, wherein said polypeptide Y comprises a portion of the constant region of an IgG isotype immunoglobulin selected from IgG1, IgG2, IgG3 and IgG4.

22. A fusion protein according to claim 21, wherein said portion of the constant region of an immunoglobulin is an IgG1 isotype immunoglobulin.

23. A fusion protein according to any of claims 1-10, in which the constant region of an immunoglobulin consists of an Fc

variant which has been modified such that the binding to a specific Fc receptor is prevented.

24. A fusion protein according to claim 23, wherein said Fc variant is an Fc variant of a human IgG1 immunoglobulin.

25. A fusion protein according to claim 24, wherein said Fc variant has substitution mutation of one or more amino acids, selected from the group consisting of E233P, L234V and L235A.

26. A fusion protein according to claim 23, wherein said fusion protein is a monomer of general formula Q_A-Y in which Q_A is a CCL2 variant selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or is a variant of CCL5, designated CCL5(S24MP25A).

27. A fusion protein according to any one of claims 1-8, of general formula Q_A-Y, in which Q_A is a chemokine or a variant thereof, and wherein the variant is either a CCL2 variant selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or is a variant of CCL5, designated CCL5(S24MP25A); and wherein the polypeptide Y comprises an Fc variant either lacking or having modifications in the residues involved in the formation of disulphide bridges.

28. A fusion protein according to claim 27 in which said Fc variant is an Fc variant of a human IgG1 immunoglobulin.

29. A fusion protein according to claim 28, wherein said Fc variant has a mutation in which the: the cysteine at positions 226 and/or 229 has been replaced with a serine and/or an alanine.

30. A fusion protein according to any one of claims 1-8,

wherein the polypeptide Y comprises an Fc variant which has been modified such that it favors the formation of heterodimers.

31. A fusion protein according to claim 30, wherein said Fc variant is an Fc variant of a human IgG1 immunoglobulin.

32. A fusion protein according to claim 31, wherein said modification consists of a "knob into hole" mutation comprising mutations in the knob chain selected from the group consisting of Y349C and T366W, and mutations in the hole chain selected from the group consisting of D356C, T366S, L368A and Y407V.

33. A fusion protein according to claim 30, wherein said fusion protein is a heterodimer of general formula $(Q_A\text{-}Y)(Q_B\text{-}Y)$, in which Q_A is CCL2 or a variant thereof selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), and Q_B is CCL5 or a variant thereof, designated CCL5(S24MP25A).

34. A fusion protein according to claim 30, wherein said fusion protein is a monofunctional dimer of general formula $Q_A\text{-}Y_2$, in which Q_A is CCL2 or a variant thereof selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A), or CCL5 or a variant thereof designated CCL5(S24MP25A).

35. A fusion protein according to any of claims 1-10, further comprising a spacer peptide between said chemokine or a variant thereof and said polypeptide Y.

36. A fusion protein according to claim 35, wherein said spacer peptide comprises the entire or a portion of the hinge region of an immunoglobulin.

37. A fusion protein according to any one of claims 1-10,

further comprising a tag peptide.

38. A fusion protein according to any one of claims 1-10, further comprising a signal peptide.

39. A nucleic acid molecule encoding a fusion protein according to any one of claims 1-10.

40. A vector comprising a nucleic acid molecule according to claim 39.

41. A host cell containing a vector according to claim 40.

42. A process for obtaining a fusion protein with activity inhibiting the binding of a chemokine to a specific receptor thereof *in vitro*, comprising the following steps:

a) generating a fusion protein comprising (i) a chemokine or a variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin according claims 1-10;

f) testing the ability of said fusion protein to inhibit the binding of said chemokine to a specific receptor thereof; and

g) selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro*.

43. A process for selecting a fusion protein having activity inhibiting the binding of a native chemokine to a specific receptor thereof *in vitro*, improved with respect to a variant of said chemokine, comprising the following steps:

a) generating a fusion protein comprising (i) a variant of a native chemokine and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an

immunoglobulin according claims 1-10;

b) testing the ability of said fusion protein to inhibit the binding of said native chemokine to a specific receptor thereof; and

d) selecting the fusion protein having an activity inhibiting the binding of said chemokine to a specific receptor thereof *in vitro* greater than said chemokine variant.

44. A pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) $Q_A\text{-}(Y)_2$ or

(d) $(Q_A\text{-}Y)_2$;

according claims 1-10 and a pharmaceutically acceptable carrier.

45. A pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) $Q_A\text{-}(Y)_2$ or

(d) $(Q_A\text{-}Y)_2$

wherein

Q_A is CCL2 or a CCL2 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is a chemokine or a variant thereof, different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin; and

a pharmaceutically acceptable carrier.

46. A pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) $Q_A\text{-}(Y)_2$ or

(d) $(Q_A\text{-}Y)_2$

wherein

Q_A is CCL5 or a CCL5 variant modified by the substitution of one or more amino acids of the N-terminal region;

Q_B is a chemokine or a variant thereof, different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin; and

a pharmaceutically acceptable carrier.

47. A pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) $Q_A\text{-}(Y)_2$ or

(d) $(Q_A\text{-}Y)_2$

wherein

Q_A is a variant of the CCL2 chemokine selected from the group consisting of CCL2(1+9-76), CCL2(P8A) and CCL2(Y13A);

Q_B is a chemokine or a variant thereof, different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin; and

a pharmaceutically acceptable carrier.

48. A pharmaceutical composition comprising a fusion protein comprising (i) a chemokine or variant thereof and (ii) a polypeptide Y comprising an optionally modified portion of the constant region of an immunoglobulin, of general formula:

(a) $(Q_A\text{-}Y)\text{-}(Q_B\text{-}Y)$;

(b) $Q_A\text{-}Y$;

(c) $Q_A\text{-}(Y)_2$ or

(d) $(Q_A\text{-}Y)_2$

wherein

Q_A is a variant of the CCL5 chemokine designated CCL5(S24MP25A);

Q_B is a chemokine or a variant thereof, different from Q_A; and

Y is a polypeptide comprising an optionally modified portion of the constant region of an immunoglobulin; and

a pharmaceutically acceptable carrier.

49. A pharmaceutical composition according to any one of claims 40-47, further comprising other additional drugs having anti-inflammatory, antitumor or antiviral activity.

50. The pharmaceutical composition of claim 48, wherein said other drugs are administered simultaneously or sequentially.

51. The use of a fusion protein according to any one of claims 1-10 in the manufacture of an anti-inflammatory, antitumor, antiviral or antiprotozoan pharmaceutical composition.

52. The use according to claim 50 , wherein said pharmaceutical

composition is an antiviral pharmaceutical composition.

53. The use according to claim 51, wherein said pharmaceutical composition is a pharmaceutical composition for the treatment of the infection caused by a human immunodeficiency virus.

54. The use according to claim 50, wherein said pharmaceutical composition is a pharmaceutical composition for the treatment of inflammatory or autoimmune diseases in which chemokine receptors are involved.

55. The use of a fusion protein according to any one of claims 1-10, in the manufacture of a composition for the diagnosis *in vivo* of diseases in which the levels of a certain chemokine are altered.

56. The use of a fusion protein according to any one of claims 1-10, in the qualitative or quantitative detection of the presence of chemokine receptors in the surface of a cell *in vitro*.

57. The use of a fusion protein according to any one of claims 1-10, in the identification of a compound with the ability to compete with said fusion protein for binding to a chemokine receptor *in vitro*.

58. A method of treating a disease or condition in which the level of any one or more of a certain chemokine are altered , comprising administering an effective amount of a fusion protein according to any one of claims 1-10 to a mammal in need of such therapy.

59. The method of claim 57, wherein the disease or condition is

selected from the group consisting of an inflammatory disease, a cancerous condition, a protozoan and a viral infection.

60. The method of claim 58, wherein the viral infection is caused by a lentivirus.

61. The method of claim 60, wherein the lentivirus is a human inmunodeficiency virus.

62. The use of a fusion protein according to any one of claims 1-10 in the manufacture of a composition for the detection of the presence of chemokine receptors in the surface of a cell *in vivo*.

63. The fusion protein, nucleic acid, vector, host cell, process, pharmaceutical composition, use, method of treatment, method of screening, or diagnostic method of any of the preceding claims wherein said fusion protein is capable of inhibiting cell migration induced by the presence of native or wild type chemokines.

FIGURE 1

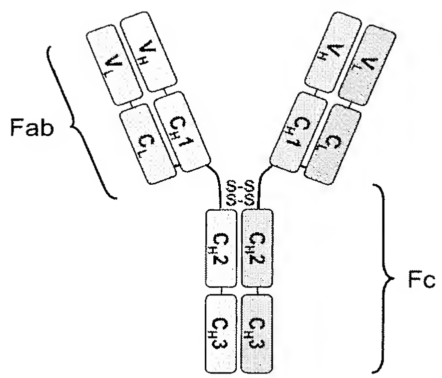
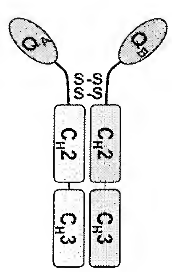
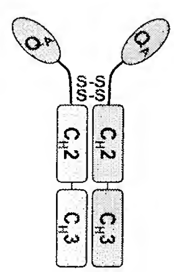


FIGURE 2

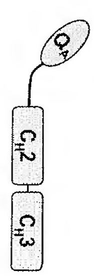
A



B



C



D

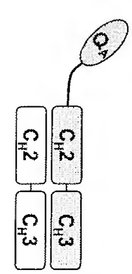
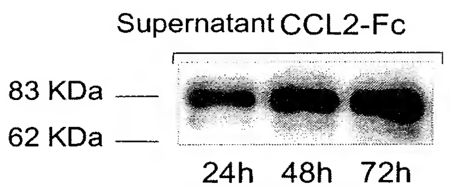


FIGURE 3

A



B

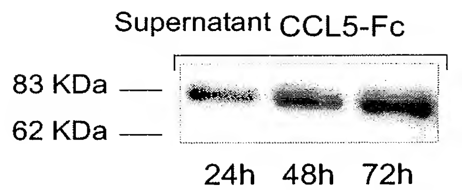
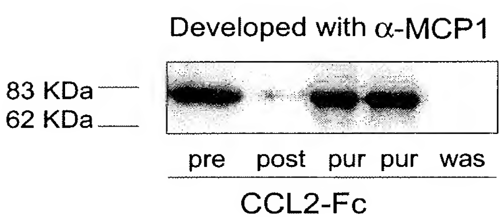


FIGURE 4

A



B

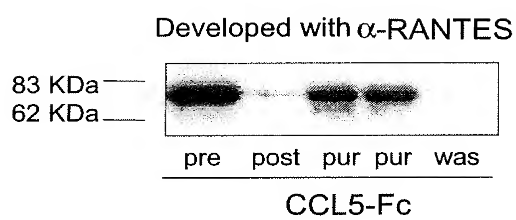


Fig 4

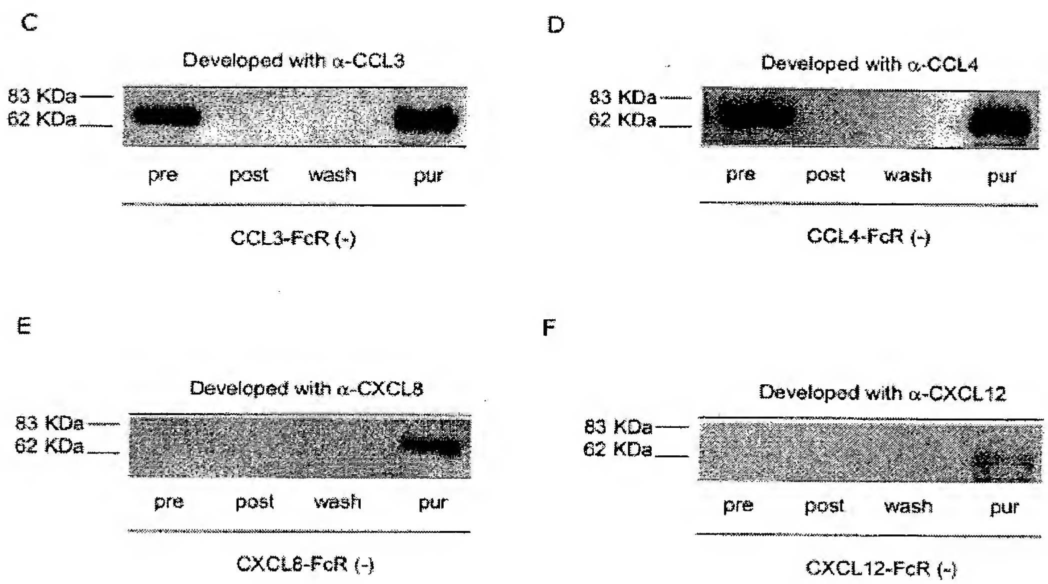

C
Developed with α-CCL3
83 KDa
62 KDa
pre
post
wash
pur
CCL3-FcR (-)
D
Developed with α-CCL4
83 KDa
62 KDa
pre
post
wash
pur
CCL4-FcR (-)
E
Developed with α-CXCL8
83 KDa
62 KDa
pre
post
wash
pur
CXCL8-FcR (-)
F
Developed with α-CXCL12
83 KDa
62 KDa
pre
post
wash
pur
CXCL12-FcR (-)

FIGURE 5

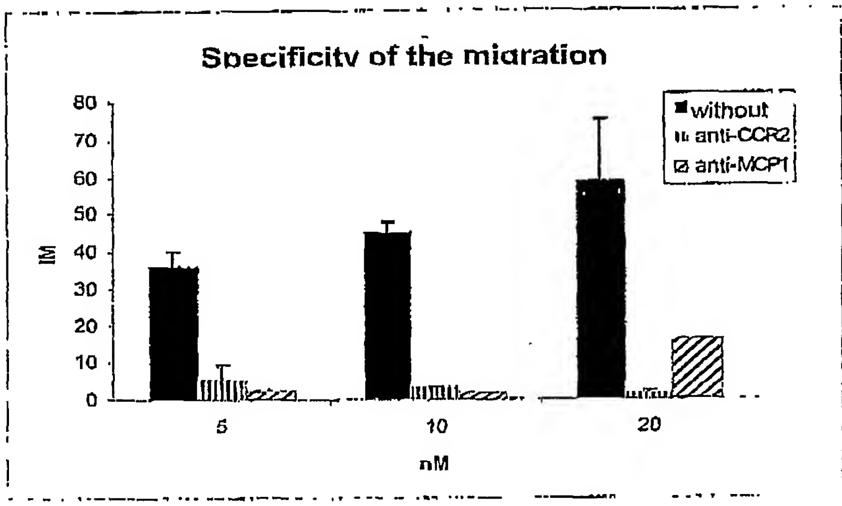
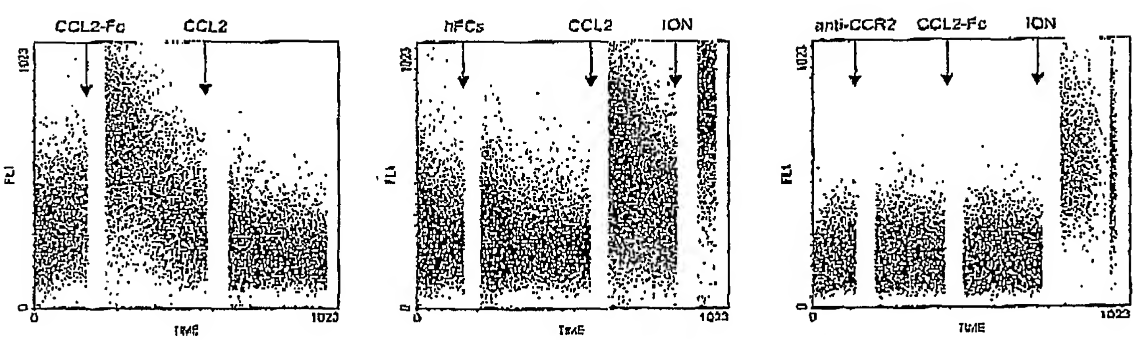


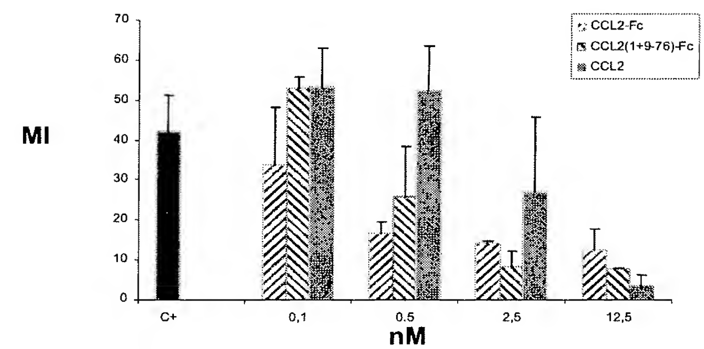
FIGURE 6



A B C

FIGURE 7

Inhibition assay against 5 nM CCL2



Inhibition assay against 2.5 nM CCL2

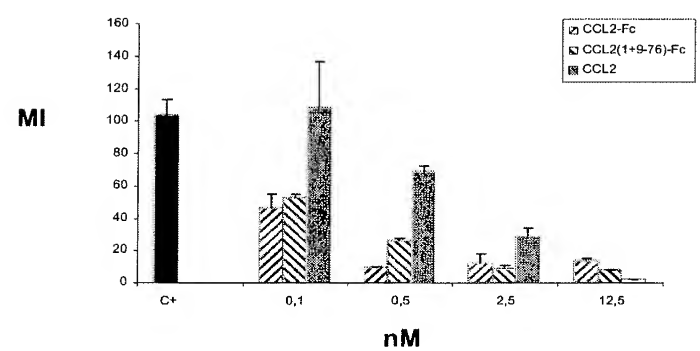


FIGURE 8

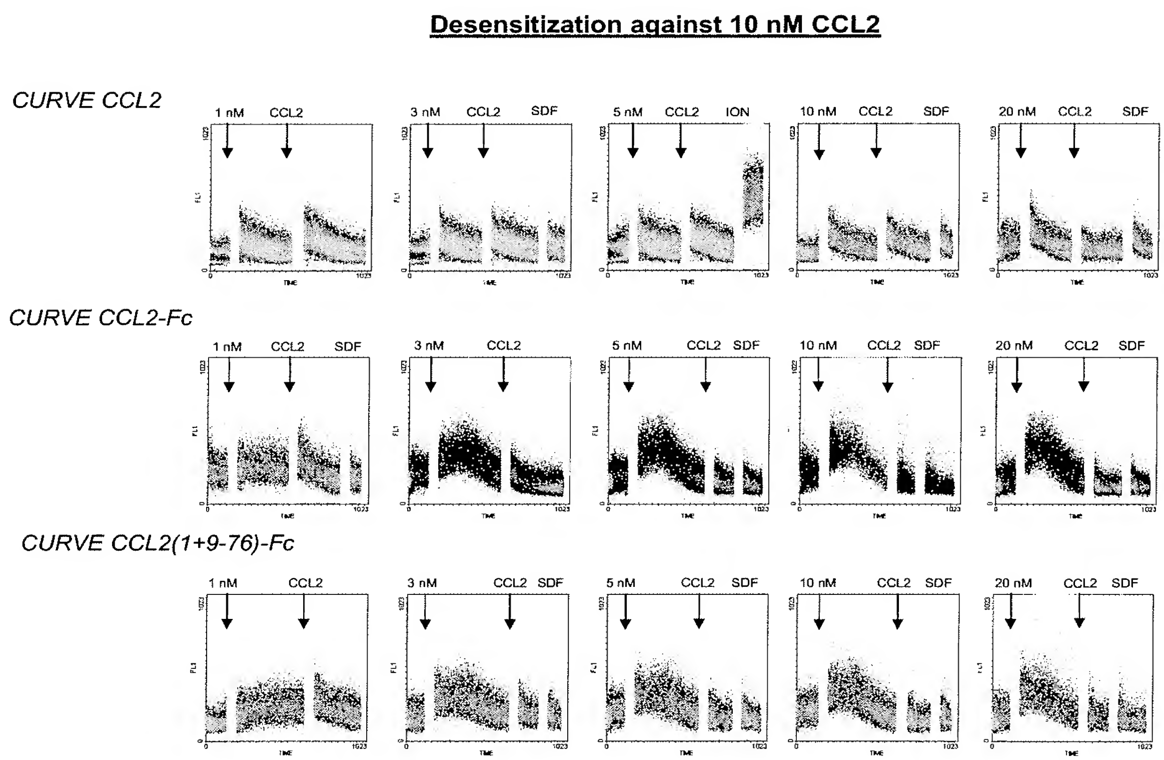
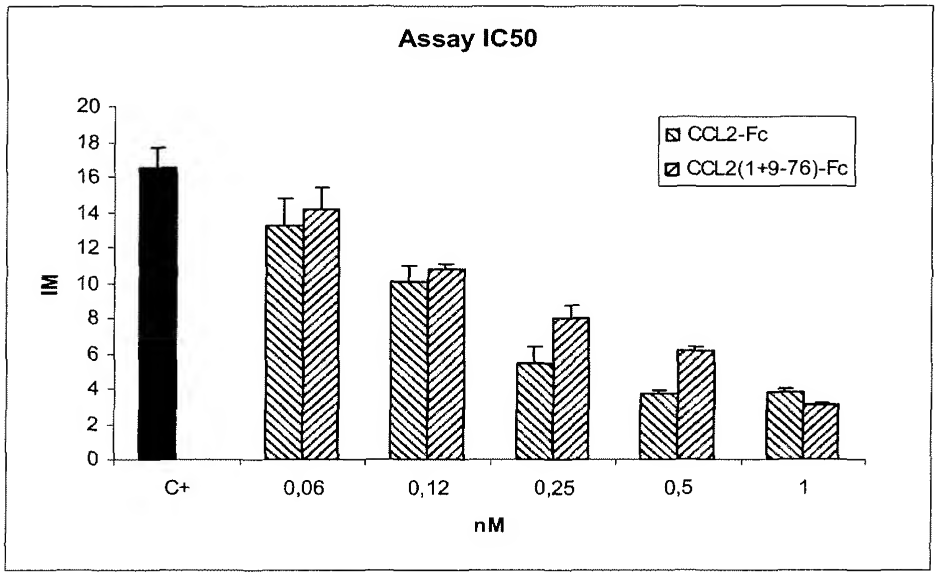

Desensitization against 10 nM CCL2
CURVE CCL2
1 nM CCL2
3 nM CCL2 SDF
5 nM CCL2 ION
10 nM CCL2 SDF
20 nM CCL2 SDF
CURVE CCL2-Fc
1 nM CCL2 SDF
3 nM CCL2
5 nM CCL2 SDF
10 nM CCL2 SDF
20 nM CCL2 SDF
CURVE CCL2(1+9-76)-Fc
1 nM CCL2
3 nM CCL2 SDF
5 nM CCL2 SDF
10 nM CCL2 SDF
20 nM CCL2 SDF

FIGURE 9

A


Assay IC50
IM
20
18
16
14
12
10
8
6
4
2
0
CCL2-Fc
CCL2(1+9-76)-Fc
C+
0,06
0,12
0,25
0,5
1
nM

B

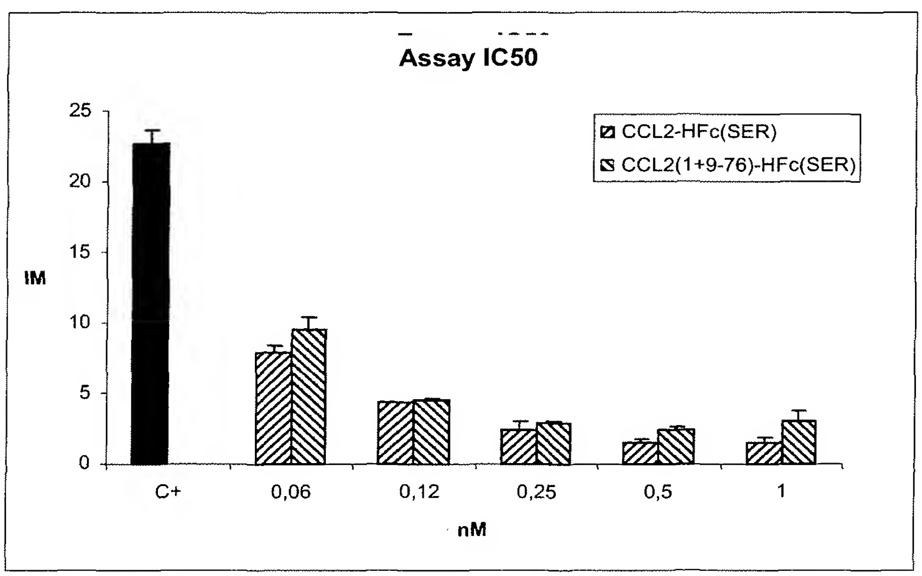

Assay IC50
IM
25
20
15
10
5
0
CCL2-HFc(SER)
CCL2(1+9-76)-HFc(SER)
C+
0,06
0,12
0,25
0,5
1
nM

Figure 9C

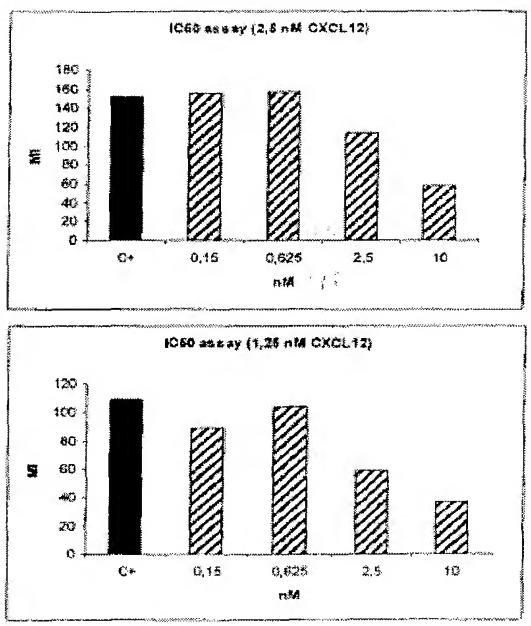


Figure 9D

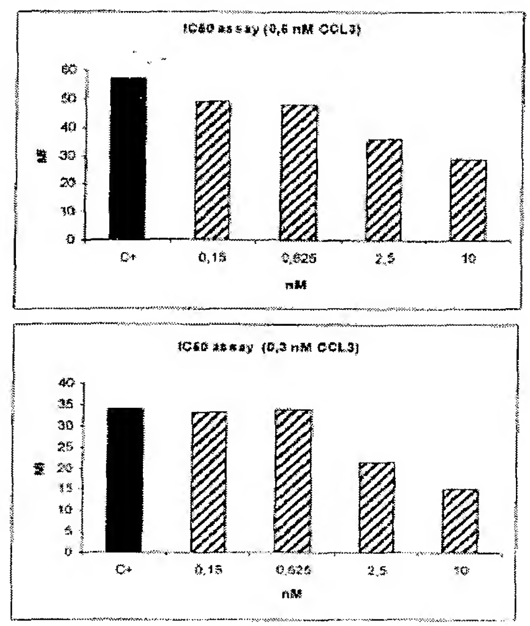


FIGURE 10

CCL2 (SEQ ID NO: 84)
QPDAINAPVT CCYNFTNRKI SVQRLASYRR ITSSKCPKE AVIFKTIVAK EICADPKQKW
VQDSMDHLDK QTQTPKT

CCL2(1+9-76) (SEQ ID NO: 85)

2-8
Q ∇ VTCCYNFTN RKISVQRLAS YRRITSSKCP KEAVIFKTIV AKEICADPK QKWVQDSMDH
LDKQTQTPKT

CCL2(P8A) (SEQ ID NO: 86)

QPDAINAAVT CCYNFTNRKI SVQRLASYRR ITSSKCPKE AVIFKTIVAK EICADPKQKW
VQDSMDHLDK QTQTPKT

CCL2(Y13A) (SEQ ID NO: 87)

QPDAINAPVT CCANFTNRKI SVQRLASYRR ITSSKCPKE AVIFKTIVAK EICADPKQKW
VQDSMDHLDK QTQTPKT

FIGURE 11

CCL5 (SEQ ID NO: 88)

MKVSAAALAV ILIATALCAP ASASPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP
AVVFVTRKNR QVCANPEKKW VREYINSLEM S

CCL5(S24MP25A) (SEQ ID NO: 89)

MKVSAAALAV ILIATALCAP ASAMAYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP
AVVFVTRKNR QVCANPEKKW VREYINSLEM S

Figure 12

CCL2-Fc (SEQ ID NO: 1)/ **CCL2(P8A)-Fc** (SEQ ID NO: 3)/ **CCL2 (Y13A)-Fc**(SEQ ID NO: 4)/ **CCL2-FcR(-)** (SEQ ID NO: 5)/ **CCL2(P8A)-FcR(-)** (SEQ ID NO: 7)/ **CCL2 (Y13A)-FcR(-)**(SEQ ID NO: 8)/ **CCL2-HFc(ALA)**(SEQ ID NO: 9)/ **CCL2-HFc(SER)**(SEQ ID NO: 10)/ **CCL2-Fc KIH-KNOB CHAIN**(SEQ ID NO: 17)/ **CCL2-Fc KIH-HOLE CHAIN** (SEQ ID NO: 18)

FUSION PROTEIN STRUCTURE:

CCL2 signal peptide: amino acids 1-23
Secreted protein: amino acids 24-99
Fc Fragment of human IgG1: amino acids 100-327

CCL2(1+9-76)-Fc (SEQ ID NO: 2)/ **CCL2(1+9-76)-FcR(-)** (SEQ ID NO: 6)/ **CCL2(1+9-76)-HFc(ALA)**(SEQ ID NO: 11)/ **CCL2(1+9-76)-HFc(SER)**(SEQ ID NO: 12)

FUSION PROTEIN STRUCTURE:

CCL2 signal peptide: amino acids 1-23
Secreted protein: amino acids 24-92
Fc Fragment of human IgG1: amino acids 93-320

CCL5-Fc (SEQ ID NO: 13)/ **CCL5(S24MP25A)-Fc** (SEQ ID NO: 14)/ **CCL5-FcR(-)** (SEQ ID NO: 15)/ **CCL5(S24MP25A)-FcR(-)** (SEQ ID NO: 16)/ **CCL5-Fc KIH KNOB CHAIN**(SEQ ID NO: 19)/ **CCL5-Fc KIH HOLE CHAIN** (SEQ ID NO: 20)

FUSION PROTEIN STRUCTURE:

CCL5 signal peptide: amino acids 1-23
Secreted protein: amino acids 24-91
Fc Fragment of human IgG1: amino acids 92-319

CCL2ps-Fc KIH KNOB(SEQ ID NO: 21)/ **CCL2ps-Fc KIH HOLE CHAIN**(SEQ ID NO: 22)

FUSION PROTEIN STRUCTURE:

CCL2 signal peptide: amino acids 1-23
Fc Fragment of human IgG1: amino acids 24-251